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A thesis submitted for the degree of Doctor of Philosophy

Investigating the pathological mechanism of neuropathy in POEMS syndrome

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Declaration

I, Stephen Keddie, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated and referenced in the thesis**.**

Abstract

POEMS syndrome (Polyneuropathy, Organomegaly, Endocrinopathy, Monoclonal gammopathy, Skin disorder) is a rare disease characterised by an inflammatory polyneuropathy and a monoclonal plasma cell dyscrasia. POEMS syndrome causes some of the most significant disability and mortality of any inflammatory neuropathy.

The pathophysiology is unknown but recognised to be cytokine mediated, notably driven by vascular endothelial growth factor, however little is known about the other mediators at play.

This thesis collates clinical data from the largest POEMS cohort in Europe in order to study the characteristic disease features, optimise therapies and identify factors that influence outcome. Utilising our POEMS sample biobank, we carry out highly sensitive immunoassays to study the cytokines released in POEMS syndrome, and whether they correlate with disease activity. We go on to study the proteome of POEMS syndrome through mass spectrometry, to uncover the biological pathways involved and identify a number of novel, potentially pathogenic molecules. Fluid biomarkers of neuropathy in POEMS syndrome and related neuropathies are additionally explored. The development and optimisation of a homebrew immunoassay for peripherin, a peripheral nerve specific biomarker is detailed. The potential clinical utility of this biomarker is compared against that of serum neurofilament light. Finally, we attempt to model the neuropathogenesis of POEMS neuropathy *in vitro* using a novel human induced pleuripotent stem cell derived neuronal culture system.

Impact statement

University College London Hospital (UCLH) established the POEMS syndrome clinic in 1996 and carried out their first autologous stem cell transplantation in 1998. The clinic provides expert multi-disciplinary, quaternary level advice to clinicians across the UK regarding the diagnosis and management of patients with POEMS syndrome. This unique environment has enabled us to produce high quality laboratory and clinical research to understand more about this rare, highly disabling disease.

This POEMS research project was inspired by the need to improve patients' outcomes through better understanding of pathogenesis, improving recognition through early diagnosis and expanding the way we measure and record peripheral nerve injury. This thesis presents the largest longitudinal cohort of POEMS syndrome cases in Europe. Through deep phenotyping and analysis, further insights into the natural history of disease including newly discovered diagnostic features,[1,2] prognostic markers, [3] and optimal therapeutic strategies are presented. [4] This work enables clinicians to provide patients with a secure diagnosis, accurate information on prognosis and the best opportunity for prolonged disease remission.

In addition to the clinical data, I have established a longitudinal collection of matched serum and plasma from disease onset, treatment, relapse and remission in POEMS syndrome and related inflammatory neuropathies. The POEMS registry is now an established resource available for ongoing research. This has already been utilised by researchers in the field to answer important clinical and scientific questions.

The mainstay of decision making when regarding therapeutics in all forms of inflammatory neuropathy is typically following the identification of neuronal disease activity. In this thesis, I have identified a blood-based biomarker of neuronal injury highly relevant to the underpinnings of peripheral nerve pathology. This is the first blood based peripheral nerve specific biomarker of neuronal tissue damage and should stimulate a body of research investigating its application and utility in peripheral nerve diseases.

Identifying the underlying pathogenesis of POEMS syndrome may help explain the almost baffling multi-system features of disease, including the characteristic neuropathy. The current mainstay of therapy in POEMS syndrome is directed towards stem cell ablation. Identification of alternative pathological pathways and disease drivers may enable treatment with more focussed therapeutics resulting in greater efficacy and less toxicity. This thesis uncovers more about the complicated inflammasome of POEMS syndrome and identifies potential key markers of disease and pathogenesis. Lastly this thesis utilises a novel experimental method studying the effect of potential pathological markers on human induced pluripotent stem cell derived neurones myelinated by rat Schwann cells. This methodological approach offers a framework for evaluating a range of peripheral nerve pathologies.

Rare diseases are not insignificant to those suffering from them. The opportunities to educate patients, carers and other clinicians in haematology and neurology has increased awareness, identification of cases and referral rates. In doing so this enables

earlier diagnosis, initiation of treatment and better outcomes. We have demonstrated this not only impacts on patients' lives but also improves on cost. [5] A priority of this research project was therefore to educate clinicians, patients, their families and the public about POEMS syndrome, and we have done this through podcasts, patient support groups and holding a very successful POEMS syndrome patient day.

From the first patient in 1996 to the present day the POEMS service has evolved. Through collaborative efforts undertaken during this research project we have developed international clinical reputation, a significant publication record and national public involvement making us one of the world leaders in the field.

Acknowledgements

First and foremost, I thank all the POEMS syndrome patients and family members who have agreed to enrol in our research programme, providing their time, information and vital biofluids for the purpose of scientific exploration, with a hope to one day improve the lives of other patients.

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and more specifically for approving our project which may have been considered too niche for non-neurological funding bodies.

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Although my time at Oxford was curtailed due to the COVID-19 pandemic, Simon Rinaldi has regardless been a huge help introducing me to his laboratory techniques, interpreting data and editing papers. Experiencing the life of a true clinician scientist has additionally been an invaluable opportunity thinking about my future career.

To my wonderful parents who give their all showing unconditional interest, support, guidance and encouragement. And despite them both promising me they will read this thesis despite having zero medical experience, I won't hold them to it! Lastly, to my wife Laura and our beautiful little children Sebastian and Isabelle who bring me endless joy and to whom I owe everything. This thesis is dedicated to them.

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Abbreviations

AA: Amino acids

ABN: Association of British Neurologists

ALS: Amyotrophic lateral sclerosis

ASCT: Autologous Stem Cell Transplantation

BSA: Bovine serum albumin

CIDP: Chronic Inflammatory Demyelinating Neuropathy

CMT: Charcot-Marie-Tooth

ddH_20: Distilled water

ECL: Electrochemiluminescence

ELISA: Enzyme Linked Immunosorbent Assay

GBS: Guillain-Barré syndrome

HFGS: Hughes function grade score

HiPSC: Human induced pluripotent stem cell

HPO: Human phenotype ontology

IAN: Idiopathic axonal neuropathy

IF: Immunofixation

LLOD: Lower limit of detection

NFL/M/H: Neurofilament light/medium/heavy

NHNN: The National Hospital for Neurology and Neurosurgery

MRI: Magnetic Resonance Imaging

MSD: Meso Scale Discovery

PBS: Phosphate buffered solution

PET: Positron Emission Tomography

PNS: Peripheral nervous system

POEMS: Polyneuropathy, Organomegaly, Endocrinopathy, Monoclonal Gammopathy, Skin

RADAR: Rare diseases ascertainment and recruitment

SIMOA: Single Molecule Array

SPE: Serum protein electrophoresis

UKAS: United Kingdom Accreditation Service

1. Introduction

1.1 POEMS syndrome

The first description of POEMS syndrome was in 1956 by R.S Crow in Bristol Royal Infirmary, published in the British Medical Journal. [6]. Crow described two patients, both with painful sensorimotor polyneuropathy and additional striking features of skin changes, clubbing, white fingernails and peripheral oedema. They were subsequently found to have a plasma cell dyscrasia, and improved following myeloma directed therapy. A number of other papers then went on to further demonstrate this association before a series of 102 cases were described in Japan, [7] who cited Professor Fukase as identifying the disease at a medical conference in 1968 and hence designated the condition the 'Crow-Fukase' syndrome. The disease describing acronym was coined by Bardwick in 1980 to characterise the clinical phenotype of a rare and disabling multisystem disease of polyneuropathy, organomegaly, endocrinopathy, M-protein and skin lesions as POEMS syndrome. [8] Cohort studies have revealed several other characteristic findings, including papilloedema, extravascular volume overload, sclerotic bone lesions and thrombocytosis (abbreviated to 'PEST' as a supplementary aide memoire). [9] Dispenzieri et al. proposed the widely accepted diagnostic criteria in 2003 in an attempt to standardise the number of features and investigation findings necessary to make a diagnosis of POEMS syndrome, [9] documented below in table 1.

Table 1.1: Diagnostic criteria of POEMS syndrome

Criteria	
Mandatory major criteria	1. Polyneuropathy 2. Monoclonal plasma cell proliferative disorder
Other Major criteria (one required)	3. Castleman disease 4. Sclerotic bone lesions 5. Raised Vascular endothelial growth factor
Minor criteria (one required)	6. Organomegaly (spleen/liver/ lymph nodes) 7. Extravascular volume overload 8. Endocrinopathy (adrenal, thyroid*, pituitary, gonadal, parathyroid, pancreatic*) 9. Skin changes 10. Papilloedema 11. Thrombocytosis/polycythemia
Other useful features	Clubbing, weight loss, hyperhidrosis, pulmonary hypertension/ restrictive lung disease, thrombotic diathesis, diarrhoea, low vitamin B_{12}.

Significant contributions to the diagnosis and management of POEMS have been made over a number of years through the work of the Dispenzieri (USA), Kuwabara (Japan) and Li (China) groups alongside other important smaller groups in France and Italy. These include the definition of core sets of diagnostic criteria, [9] the identification of VEGF as a major biomarker for the disease, [10–13] contributions to epidemiology and diagnostic findings, [7,9,14–21] as well as a number of studies informing current treatments and outcomes. [7,17-33]

1.2 Characteristic features of POEMS syndrome

1.2.1 Polyneuropathy

The clinical phenotype of POEMS syndrome is often diverse, reflecting the multisystem involvement, but is typified by the presence of a peripheral neuropathy which is the most disabling feature. Early in the disease there is seldom proximal weakness and throughout the disease course striking distal weakness of the hands and feet predominates. [39] A typical POEMS neuropathy is a symmetrical, sensorimotor, length-dependent, painful neuropathy which progresses proximally and is resistant to plasma exchange and IVIG. [7,9,15]

Neurophysiology in POEMS syndrome is characterised by conduction velocity slowing, and is therefore classified as demyelinating in nature, and fulfils diagnostic criteria for the more common cause of demyelinating neuropathy, Chronic Inflammatory Demyelinating Polyradiculoneuropathy (CIDP) in 60-70% of cases. [40] CIDP is characteristically heterogeneous in clinical presentation with several subtypes, [41] and therefore commonly, neurophysiology demonstrating demyelination is misinterpreted

as being CIDP regardless of the clinical phenotype. Studies have attempted to distinguish the neurophysiological characteristics of POEMS syndrome from CIDP. Slowed conduction velocity combined with marked early axonal loss is typical for POEMS syndrome. Motor conduction velocities (MCV) are equally reduced in the upper and lower limbs, and velocities are often slower than CIDP. Uniform demyelination along the nerve with no conduction block (10% compared to 45% in CIDP), [42] normal terminal latency indices (TLi) and F-wave delay proportionate to MCV slowing, as opposed to focal involvement of selected fibres in CIDP, is the norm.[42–45] At diagnosis, approximately 50% of nerves with reduced motor conduction velocity MCV have reduced CMAP amplitude demonstrating the early axonal loss. [45] CMAPs in the lower limbs are more severely affected than the upper limbs, with abnormal values in 96% peroneal, 97% tibial, 65% ulnar and 67% median nerves when tested in a cohort of 138 cases. [43] Sural sparing is not seen in POEMS syndrome as it sometimes is in CIDP. [43]

POEMS exhibits albuminocytological dissociation on cerebrospinal fluid analysis, which may provide insights to pathogenesis of the neuropathy. Imaging of the nerve roots displays smooth thickening and enhancement. [46] Such findings are also seen in CIDP and thus do not differentiate the two conditions. Identification of clear discerning objective features or biomarkers separating POEMS neuropathy from CIDP are crucial in improving early diagnosis and enabling initiation of disease specific treatment.

1.2.2 Monoclonal gammopathy

Monoclonal gammopathies are conditions in which an abnormal production of antibodies occurs by monoclonal expansion of bone marrow plasma cells. Identification of a monoclonal gammopathy can be through serological and urine testing for serum protein electrophoresis (SPE), immunofixation and serum free light chain (SFLC) analysis, bone marrow or plasmacytoma histopathology. Monoclonal gammopathies are determined by the antibody heavy and/or light chain isotype. The majority of individuals (70%) develop IgG gammopathies, followed by IgM (10-20%) and then IgA (10%).[47] When there are no systemic or infiltrative features, serum protein is under 3g/dL and bone marrow histology demonstrates under 10% plasma cells, such monoclonal gammopathies are classified as monoclonal gammopathies of unknown significance (MGUS). However, monoclonal gammopathies can also indicate evidence of more sinister haematological pathology such as POEMS syndrome or haematological malignancies such as Waldenströms macroglobulinaemia and myeloma.

POEMS syndrome is a haematological condition on a spectrum of malignancy and characterised by a monoclonal lambda light chain restricted plasma cell disorder. This appears to be the underlying driver of disease, resulting in systemic inflammation, multi-organ involvement including neuropathy, vascular leak and in some cases, death. For reasons unknown, gammopathies in POEMS syndrome are characteristically IgA and IgG lambda light chain restricted (52 and 47% respectively), and almost never IgM or kappa light chain. [9] This is in contrast to the majority of monoclonal gammopathies found in association with neuropathy which are IgM (78%) and kappa (88%), and in other paraproteinemic neuropathies such as anti-MAG, in which IgM is universal. [48]

1.2.3 Multi-system features

The wide-ranging multi system features of POEMS are displayed in table 1 and will be discussed in further detail in chapter 2. Endocrine disturbance was noted but not quantified in reported cohorts. Organomegaly of the spleen, liver or lymph nodes is detected in around 50%.[7,9,14] Skin manifestations such as glomerular haemangiomata, acrocyanosis, hypertrichosis, nailfold changes and clubbing are present in 70-90%. [9,14] Papilloedema is rare in inflammatory neuropathy so when discovered should trigger investigation for POEMS syndrome, where it is seen in around 40%. [9,49] Capillary leakage also occurs, causing peripheral oedema, but can also occur in the lungs, abdomen and heart resulting in poor gas transfer, ascites and pericardial effusions in severely affected patients. This capillary leakage is very common in POEMS syndrome and thought to be an effect of VEGF on blood vessel permeability. Bone lesions are commonly present (up to 97%), and characteristically defined as lytic with a sclerotic rim.[50] Thrombocytosis, polycythaemia, and arterial and venous thromboembolism are seen in active disease and also as a result of therapy associated complications.

As is typical in medicine, textbook definitions very rarely reflect what is seen in clinical practice. Although clear diagnostic criteria exist, POEMS syndrome remains notoriously difficult to diagnose. Despite the presence of a monoclonal lambda light chain plasma cell dyscrasia in 95% of cases [51] coupled with the highly sensitive biomarker VEGF, [11] misdiagnosis remains likely. The heterogeneity of clinical presentations, with patients commonly presenting with only a limited number of the features described, contributes to the diagnostic difficulties. The subtlety or lack of bone marrow abnormality provides false reassurance of the absence of a monoclonal plasma cell disorder. The typically

inflammatory neuropathy mimics that of the much more familiar chronic inflammatory demyelinating polyneuropathy (CIDP). Features such as endocrinopathy, papilloedema and skin conditions are non-specific, and may not typically be attributed to a neurological syndrome. Furthermore, investigations may be misinterpreted or down-played, resulting in reports which may not synthesise the whole picture. With the diagnostic uncertainty and misleading information in such a complex disease, it is little wonder clinicians fail to consider the diagnosis.

1.3 Investigative workup of POEMS syndrome

To reveal the full range of diagnostic features of POEMS syndrome, a large range of investigations are often required and are summarised in table 1.2.

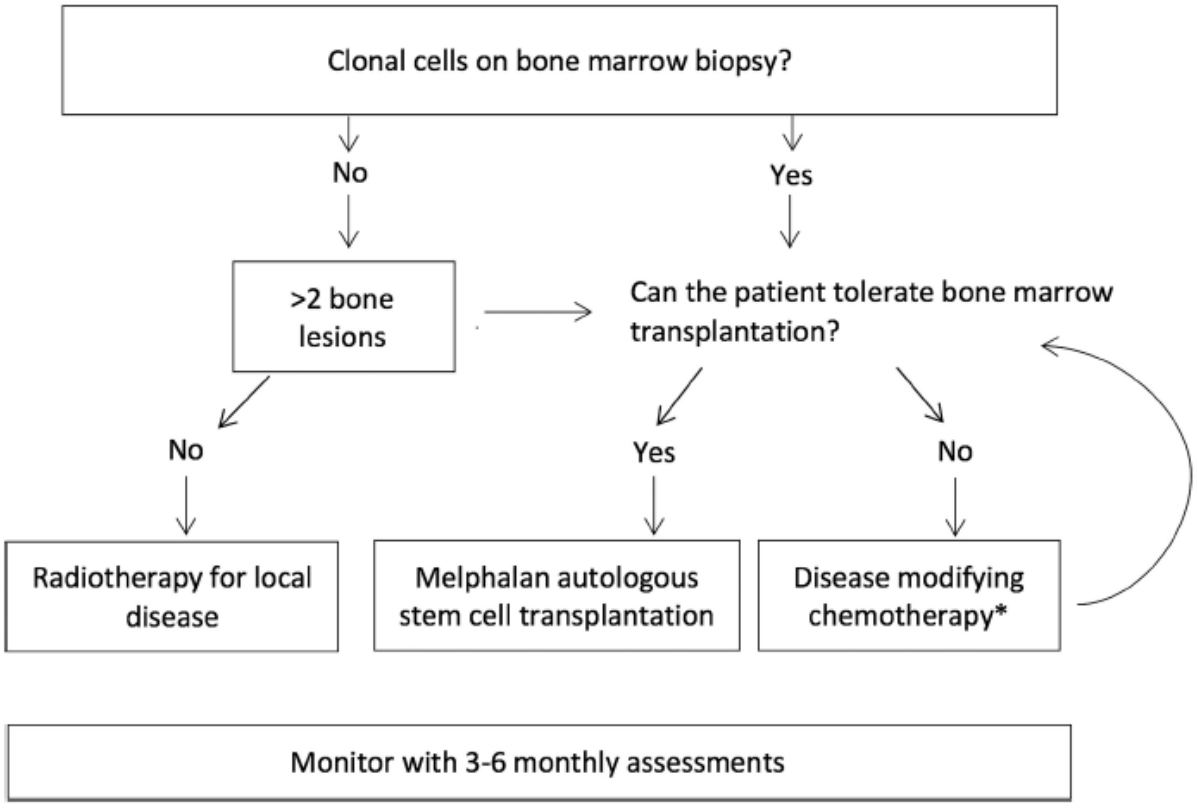
Table 1.2: Investigations required for POEMS syndrome diagnosis. Taken from Keddie et al "POEMS neuropathy: optimising diagnosis and management".[46]

Feature	Investigation		Typical abnormality
Polyneuropathy	NCS/EMG		Axonal and demyelinating. Preferential lower limbs, intermediate > distal nerve CV slowing. Conduction block (6%) and temporal dispersion unlikely.
	Nerve biopsy		Not necessary if diagnosis is clear with elevated VEGF levels. Axonal degeneration, diffuse myelinated fibre loss, increased epineurial blood vessels. Uncompacted myelin lamellae.
	Cerebrospinal fluid		Albuminocytological dissociation. Normal cell count. Mild increase opening pressure. Not specific so not always necessary.
Organomegaly	CT chest/abdomen/pelvis and PET CT		Lymph node, spleen, liver
Endocrinopathy	Adrenal	Cortisol	Typically low- Addisonian
	Thyroid	TSH, T4	Hypo or hyperthyroid
	Pituitary	LH, FSH, IGF-1, ACTH, Prolactin	Typically hypofunctioning
	Gonadal	Testosterone, oestradiol	Typically low
	Parathyroid	PTH	
	Pancreatic	HbA1c, glucose	Typically raised
Monoclonal plasma cell disorder	Serum protein electrophoresis		IgG or IgA lambda monoclonal, low kappa:lambda ratio.
	Immunofixation		
	Serum free light chain analysis		
	Urine protein electrophoresis/immunofixation		Bence Jones protein
	Bone marrow biopsy +/- targeted bone lesion biopsy		Presence of plasma cells on immunofixation, typically lambda light chain restricted
Skin	Clinical diagnosis; history and examination		Acrocyanosis, hypertrichosis, nail changes, glomerular haemangiomata
Papilloedema	Ophthalmological assessment		
Extravascular volume overload/ cardiac involvement	Echocardiogram		Reduction of left or right ventricular ejection fraction, elevation of pulmonary artery pressure. Evidence of previous ischemia.
Sclerotic bone lesions	CT bone windows, PET CT imaging		Sclerotic lesions / mixed lytic with sclerotic
Thrombocytosis	Full blood count		Increased platelets
Pulmonary function	Pulmonary function tests		Pulmonary hypertension, restrictive disease, respiratory muscle weakness, reduced diffusion capacity.

1.4 Treatment and outcomes

POEMS is a treatable disease. Treatment options are relatively limited but effective, with simple algorithms to guide decision making. [52] Treatment of POEMS is aimed at the site and degree of clonal haematologic disease (see figure 1.1), including supportive care largely for the neurological consequences.

Figure 1.1: Treatment algorithm in POEMS syndrome. Taken from Keddie et al "POEMS neuropathy: optimising diagnosis and management".[46]



1.4.1 Focal disease

Focal disease is determined by normal histology on pelvic bone marrow biopsy, where the abnormal plasma cell clone is limited to less than 3 focal plasmacytomas. Radiotherapy at doses of 40-50Gy over approximately four weeks at a rate of 1.8-2 Gy per fraction is often effective in focal disease, and often curative. [53] Ten year overall survival of 91 patients treated at the Mayo Clinic with radiotherapy, each of whom had between 1 and 3 lesions, was 70 %. [23]

1.4.2 Generalised disease

If abnormal plasma cells are detected on bone marrow biopsy, this signifies generalised disease. Autologous stem cell transplant (ASCT) is the gold standard for patients with sufficient cardio-respiratory reserve and performance status. Those not deemed fit for ASCT should be treated with chemotherapeutic regimens such as cyclophosphamide, thalidomide, melphalan or lenalidomide often in combination. [54]

1.4.2.1 Autologous stem cell transplantation

Autologous stem cell transplantation (ASCT) is the treatment of choice where radiotherapy is not suitable and where the physical performance status of the patient allows. Priming with dexamethasone, thalidomide or lenalidomide or combinations may protect against acute engraftment syndrome. [31,36,55] Engraftment syndrome occurs 7-15 days after transplant and comprises fluid overload and weight gain, respiratory compromise, skin eruptions and diarrhoea and can be fatal. [56] Most ASCT is performed

as Melphalan 140- or 200-Conditioned regimens, and has the highest progression free and overall survival from all the treatment modalities; five year overall survival is reported at 90-95%, and five year PFS at 63-76%. [22,23,31,32,36] Studies have demonstrated reduced rates of engraftment syndrome with 4 days of 40 mg dexamethasone, [54] or lenalidomide between stem cell collection and ASCT. [22]

1.4.2.2 Chemotherapy

Systemic chemotherapy can either be used as a long-term treatment, or as a bridging therapy to optimise patients for ASCT at a later date. There are no established guidelines for exactly which chemotherapy to choose or which is most efficacious, but melphalan, cyclophosphamide, thalidomide, bortezomib and lenalidomide are used mostly. Each therapy including outcomes and complications have been reviewed in detail in a separate publication.[57]

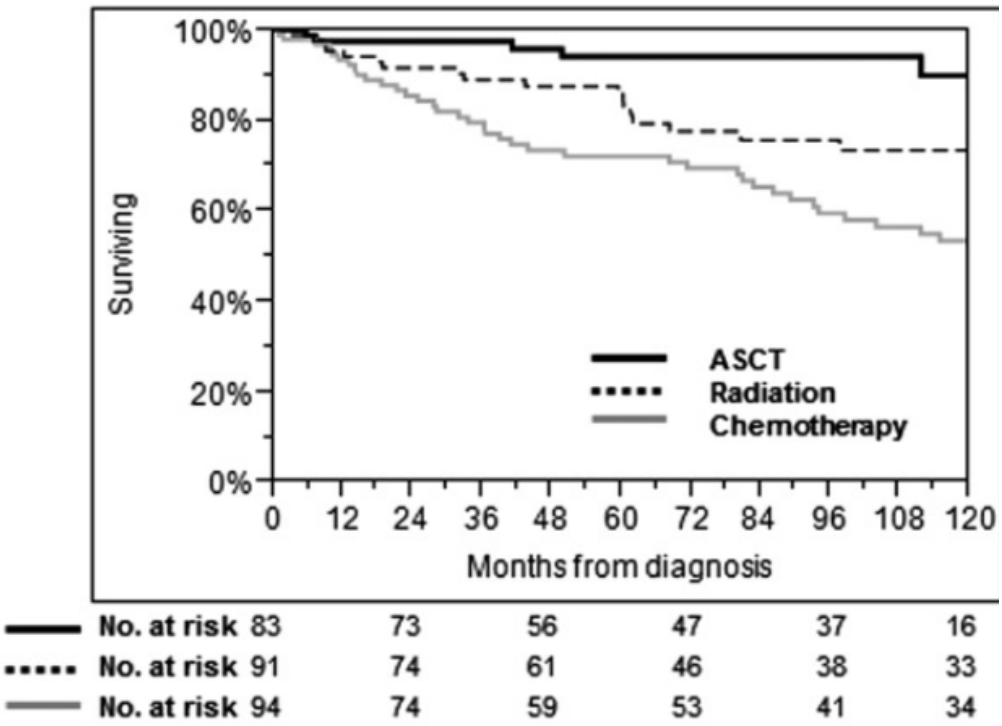
It is however worth noting that lenalidomide appears to be the most efficacious chemotherapeutic or immunomodulatory agent in POEMS syndrome to date, with reports of rapid resolution of volume overload and VEGF, improvement of neuropathy and haematological response. A systematic review of lenalidomide in 51 POEMS patients demonstrated at least very good partial response in 58% and partial response in 37%. VEGF was reduced in all cases, and neuropathy improved in 92%. [58] Li *et al.* studied 138 patients with POEMS and ASCT; of those, 32 patients initially deemed unfit for ASCT were treated with lenalidomide which made subsequent ASCT feasible. [31]

1.4.3 Prognostic factors and outcome

Untreated, the median survival of POEMS syndrome is 33 months, but recent studies of 138 and 362 patients from two large series have demonstrated 5 year survival rates of 79% and 84% respectively. [23,33]

Kourelis *et al.* studied the Mayo Clinic's outcome data of 291 POEMS patients, and identified three factors associated with superior overall survival (OS); younger age, serum albumin > 3.2 g/dL and complete haematological response following treatment. [23] Wang *et al.* have developed a prognostic nomogram for POEMS patients, in which OS and progression free survival (PFS) can be estimated through combining patient age, evidence of pulmonary hypertension, pleural effusion and estimated glomerular filtration rate. [33] Kourelis and Dispenzieri validated the nomogram in their Mayo cohort, suggesting it has external validity. [38] Misawa *et al.* demonstrated normalisation of VEGF at 6 months post treatment correlated with prolonged relapse free survival, improved grip strength and CMAP amplitudes at 12 months.[59] Zhao *et al.* have also cited the prognostic value of VEGF response alone, and in combination with haematological response, as a surrogate endpoint in clinical trials. [13]

Figure 1.2: Overall survival relative to treatment type in POEMS syndrome in 291 patients. Taken from Kourelis et al. [23]



1.5 Epidemiology

POEMS syndrome is a rare disease, and the exact incidence and prevalence are unknown. Studies reporting the largest cohorts of POEMS patients are in America (291 patients) [23] and China (476 patients). [13] Other studies over 5 years old described 102 patients in Japan, [7] 25 in France, [15] and 29 in India. [60] A recent update has been provided in Japan which estimate 392 cases and a prevalence of 0.3 per 100,000 from a nationwide survey with a 52% response rate, thus the certainty of this estimate is moderate. [61] A literature based cohort study in 2019 recorded 1946 cases of POEMS syndrome in China, however the methodology of identifying cases through searching national databases would undoubtably result in duplication and inaccuracies and thus this prevalence should be interpreted with caution. [62] Figures are unlikely to accurately depict the true epidemiology, but do suggest POEMS is possibly more common in China and Japan than Europe and the USA. The onset of POEMS most frequently occurs in the fifth or sixth decade and is about 2 ½ times commoner in men than women. [9,61]

1.6 Pathophysiology of POEMS syndrome

The pathophysiology of POEMS syndrome remains poorly understood. The monoclonal IgA or IgG lambda light chain restricted plasma cell disorder inherently appears to be the underlying driving force of disease. These aberrant plasma cells are thought to contribute towards a co-existing upregulation of pro-inflammatory cytokines, resulting in a host of downstream multi-system effects including neuropathy, however there are few data to support such findings. These three pathological processes will be discussed in more detail below.

1.6.1 Monoclonal plasma cells

Little is known about the functional and pathogenic role of the plasma cells in POEMS syndrome. Of notable interest is that detectable monoclonal antibodies from plasma cells are predominantly Immunoglobulin A (IgA) or IgG, and lambda light chain restricted in over 95% of cases. [9,52] There are currently no known mechanisms for the highly selective immunophenotype inherent to POEMS. Histopathological specimens taken from nerves, lymph nodes, kidneys or meninges for example do not demonstrate deposits of plasma cells, their antibodies, cryoglobulins (very rarely) or amyloid. Such findings demonstrate that plasma cells are inherent to POEMS syndrome pathogenesis, but do not cause end organ damage.

In the literature, POEMS syndrome is most often compared to that of the more prevalent multiple myeloma, in which substantially more knowledge of pathogenesis exists. Myeloma is a clonal plasma cell malignancy which is characterised by an abnormal increase of monoclonal paraprotein leading to end-organ damage including bone lesions, hypercalcaemia, renal insufficiency and anaemia. The pattern of monoclonal heavy chain distribution in myeloma is IgG in 52%, IgA 21%, IgD 2% and IgM in only 0.5%, similar to that of POEMS syndrome. [63,64] Although significant differences exist between the two diseases, advances in techniques to understand plasma cell cancer biology and the mechanisms of malignant transformation may be appropriated from myeloma research to develop our understanding of the complex pathological mechanisms leading to disease in POEMS syndrome.

Four very early and partially overlapping molecular pathologic events are shared by MGUS plasma cells and myeloma tumours, which provide some insight towards the process of transformation of MGUS to dangerous plasma cell clones; IgH translocations, aneuploidy, chromosome 13 deletion and dysregulation of the cyclin D gene. [65] Secondary translocations, copy number variants, oncogenic mutations and epigenetic alterations also drive clonal evolution and expansion from MGUS to malignant clones. [66] Again, although such findings have not been demonstrated in POEMS syndrome, research looking into the pathogenesis of POEMS syndrome focussing on the monoclonal disease should focus on uncovering similar disease processes.

A number of common cytogenetic abnormalities found in multiple myeloma are also associated with POEMS causation and prognosis. The 14q32 IgH translocation has been observed in 45% cases of POEMS syndrome, and included the common t(4;14) and t(11;14) translocations seen in myeloma. [21] Bryce et al confirmed 13q deletion, a typical aberration in myeloma, was also widely present in plasma cells of POEMS patients.[67] Furthermore, of the 81 functional immunoglobulin light chain variable region genes (IGLV), there is restricted usage of IGLV 1-40 and 1-44. This is a consistent finding and has been demonstrated in 57 POEMS syndrome cases from three publications.[68–70] This phenomenon is not seen in myeloma or amyloidosis. The molecular mechanisms triggering the clonal expansion of plasma cells remain unclear, but such data suggests a degree of antigen affinity maturation. [71,72] Hypotheses have been made that this restricted expression may effect communication between plasma cells and neighbouring stromal cells in terms of altering expression of cytokines, although further work needs to be performed. [69]

Table 1.3: Cytogenetic abnormalities in POEMS syndrome compared to myeloma (MM), light chain amyloid (AL) and monoclonal gammopathy of undetermined significance (MGUS). Taken from Kang WY et al 2013. [21]

	13q14 (%)	1q12 (%)	17p13 (%)	14q32 translocation (%)	t(4;14) (%)	t(11;14) (%)	t(14;16) (%)
POEMS (current)	25.0	20.0	0.0	45.0	15.0	25.0	0.0
POEMS (6)	41.9	NA	0.0	22.6	0.0	9.7	0.0
MM (12)	50.0	40.0	10.0	50.0–90.0	15.0	15.0	6.0–7.0
AL (14, 15)	30.3–34.6	23.8	0.0–1.6	48.0–78.0	0.0–4.1	39.3–52.8	1.2–1.8
MGUS (3, 4)	22.1–30.7	14.7	2.2–4.0	26.2–59.1	1.9–9.4	18.7–26.0	1.1–2.7

NA, not assessed.

One study which looked at whole exome sequencing in POEMS identified recurrently mutated genes in EHD1, EML4, HEPHL1, HIPK1, KLHL6, LTB and PCDH10. Not a single driver gene of myeloma was identified suggesting a separate, unique genetic architecture. [73] KLHL6 expression is largely restricted to germinal centre B cells in humans, and mutations have been reported in patients with various post germinal centre B-cell neoplasms such as chronic lymphocytic leukaemia, MGUS and myeloma.[74,75] It is not clear whether the identified mutations are associated with POEMS pathogenesis since none are directly related to VEGF/ cytokine signalling or other plausible functional or pathogenic processes specific to this disease. In studying transcriptional profiles of POEMS plasma cells through RNA sequencing, upregulated genes were associated with pathways related to immune response and cell adhesion, and downregulated genes were associated with tumorigenesis pathways.[73]

To summarise, although an abnormal plasma cell dyscrasia is clearly inherent to POEMS pathogenesis, of which certain cytogenetic abnormalities appear influential, little else is known regarding the development of the plasma cell clone or its influence on inflammatory cascades which may be relevant towards end organ pathogenesis.

1.6.2 Cytokines in POEMS syndrome

Cytokines appear to play a significant role in POEMS syndrome pathogenesis. Firstly, vascular endothelial growth factor (VEGF) is a potent proangiogenic cytokine significantly raised in active POEMS syndrome. [2] The proangiogenic properties of VEGF appear consistent with multiple clinical features of POEMS syndrome such as organomegaly, skin lesions, thrombosis, papilloedema and extravascular volume overload. Alterations in pro-inflammatory cytokines such as fibroblast growth factor, hepatocyte growth factor, tumour necrosis factor, interleukin (IL) 6, IL 12, HIF1α and N-terminal propeptide of type I collagen have also been reported in POEMS syndrome,[76–79] but more research is required to understand their roles in pathogenic mechanisms. The full repertoire of cytokines implicated in POEMS syndrome, including novel data, will be presented in chapter 3.

1.6.3 Pathophysiology of neuropathy in POEMS syndrome

The process of nerve damage in POEMS syndrome is not understood. Neurophysiology in POEMS neuropathy demonstrates uniform and intermediate nerve segment damage which is both axonal and demyelinating. This uniform damage across the nerve demonstrates that the pathology is diffuse. The multi-system nature of POEMS syndrome suggests the organ damaging compounds are located throughout the systemic circulation, and may cause disease through toxic or immune-mediated inflammatory damage. In contrast, CIDP pathology for example only affects the peripheral nerve, is multifocal, and the result of more specific antibody-antigen binding.

Histopathology of nerve fibres alludes to the process of neuronal damage in POEMS syndrome, but does not necessarily shed light on the underlying pathogenesis. Marked oedema in the endoneurium has been documented, suggested to be potentially induced by upregulated VEGF and other proinflammatory cytokines. [80] Other studies have reported significant loss of myelinated fibres, nodal ion-channel disruption, segmental demyelination and axonal degeneration.[69–74] More detailed histopathology of the neuronal vasculature reveals vessels filled with platelets, and narrowing and occlusion of endoneurial capillaries. [85] None of these findings are disease specific. [86] Endoneurial IgG, IgM and Complement C3 have also been detected, which may suggest disruption to the blood nerve barrier. [85] This breakdown of the blood nerve barrier may lead to peripheral nerves being further exposed to other pathogenic molecules such as cytokines, antibodies or immune cells leading to neuronal disruption and death.

Another characteristic histopathological finding in POEMS nerve biopsies is the finding of uncompacted myelin lamellae (UML) in approximately 70% of cases. [18,81,83,84] It is uncertain whether this is a direct result of myelin or axonal damage. UML can be seen in other demyelinating neuropathies such as Charcot Marie Tooth, whereby mutations occur to P0/MPZ and PMP22 proteins which function as adhesion molecules connecting adjacent myelin lamellae. [87] The finding is limited to a number of neuropathies and thus likely reflects the underlying pathogenesis, the process of which remains undetermined.

1.6.4 Blood nerve barrier alteration in POEMS syndrome

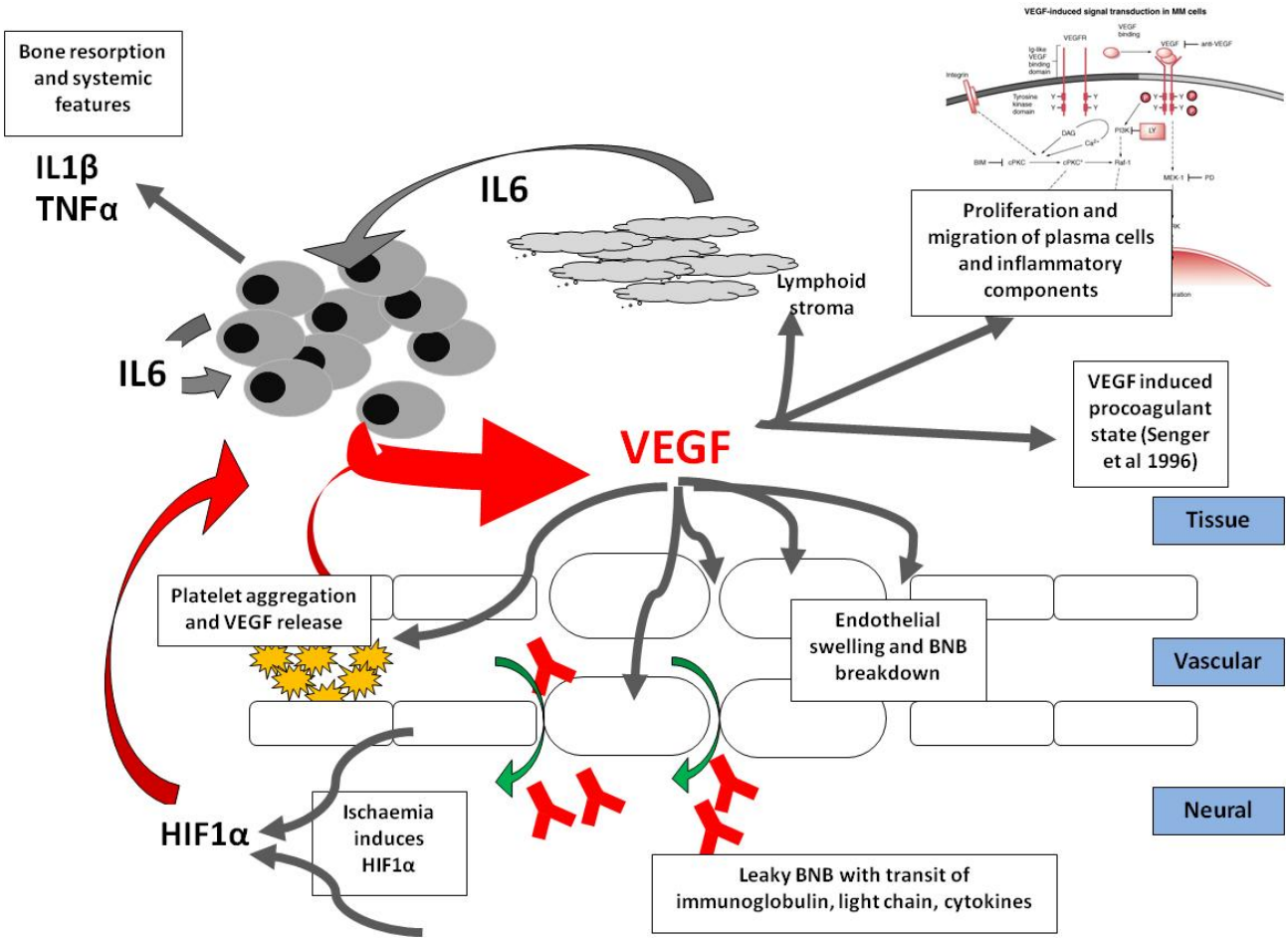
Another important consideration is how such pathogenic molecules access the peripheral nerves. The blood nerve barrier (BNB) protects the endoneurial microenvironment by restricting the movement of pathogenic T cells and various soluble factors including cytokines, chemokines and immunoglobulins from the blood to the peripheral nervous system. Compared to the blood-brain barrier (BBB), only limited knowledge has been accumulated regarding the function, cell biology and clinical significance of the BNB. Without direct evidence, it has been proposed that VEGF causes microvascular permeability at the blood nerve barrier which allows for infiltration, possibly of VEGF, other inflammatory cytokines, immune cells and possibly toxic metabolites.[88]

Endothelial cells composing small endoneurial vessels are non-fenestrated and contain few pinocytotic vesicles. They are connected by complex and continuous tight junctions which express occludin, claudin-5, claudin-12, ZO-1, ZO-2 and JAM-A which isolate the endoneurium from the intravascular component. [89] In addition, endothelial cells forming the BNB express various receptors and transporters which remove toxic metabolites to maintain neuronal homeostasis. Pericytes are polygonal cells located at the endothelial cell walls which maintain physiological BNB function, enhancing expression of endothelial tight junction molecules through secretion of various soluble factors, especially fibroblast growth factor.[90] The endothelial cells are further surrounded by a basement membrane. The BBB further contains an abluminal layer called the glia limitans perivascularis and an astrocytic endfoot layer which is not present in the BNB.[90] This structural difference may suggest the BNB would be 'weaker'

or ‘leakier’ to the BBB, however subsequent studies have demonstrated this to be untrue. [91] In POEMS syndrome and related inflammatory neuropathies, neuropathology may occur as a consequence of endoneurial microvessels losing or lacking BNB barrier properties, allowing influx of pathogenic substances to the nerve. Fenestration of endoneurial microvessels and loss of tight junctions have been demonstrated in studies of BNB function in immune mediated neuropathies. These pathological changes likely occur due to alterations of tight junction proteins. Kanda et al. interestingly have demonstrated downregulation of claudin-5, a tight junction protein, in a human BNB derived endothelial cell line from exposure to VEGF and proinflammatory cytokines such as Transforming growth factor beta (TGF-β).[92]

Neuropathogenesis in POEMS syndrome is considered to be related to BNB disruption by high circulating levels of VEGF, causing microvascular hyperplasia and endoneurial oedema. The microangiopathy may lead to local tissue hypoxia, upregulation of HIF-1α by all constituents of the nerve, causing secondary VEGF release and a toxic gain of function. Serum VEGF BNB dysfunction likely occurs throughout the entirety of the peripheral nerve, rather than being confined to areas of weakened BNB or particular antigenic epitopes, which may explain the neurophysiological findings of diffuse pathology involving the nerve trunk.[40]

Figure 1.3: Schematic diagram of the interplay between aberrant monoclonal bone marrow plasma cells, cytokines and nerve pathology in POEMS syndrome.



1.7 Research proposal

1.7.1 Rationale for study

POEMS syndrome is a rare but important cause of an inflammatory neuropathy associated with significant disability and yet favourable outcomes if treated appropriately. Misdiagnosis is common resulting in disease progression and worse outcomes. Understanding more about the natural history of POEMS syndrome and key features which separate it from more common forms of neuropathy such as CIDP is paramount to early recognition and treatment.

The role of a neurologist in POEMS syndrome is to firstly identify the typical neuropathy and its associated features to formulate the diagnosis, then to define, measure, monitor and prognosticate the extent and severity of neuropathy and related functional impairment. Current techniques for measuring peripheral nerve damage and recovery are laborious and crude. Establishing effective, reliable and sensitive biomarkers of neuropathy would therefore be of significant clinical utility and may allude to underlying peripheral nerve pathology.

Although poorly understood, the multi-system, pro-inflammatory nature of the disease suggests pathogenesis is cytokine mediated, rather than from direct B-cell effects. The lack of immune complex or light chain deposition, vasculitis or amyloid in histopathological samples (sural nerves, skin, pachymeninges) support this theory. For this reason, we intend to comprehensively study the role of cytokines in

POEMS syndrome pathogenesis, the possible interaction with VEGF, and their effect on neuropathy.

To date, disease modifying therapies are directed towards suppressing or ablating the monoclonal plasma cell population, with the gold standard of treatment being an autologous stem cell transplant (ASCT). Such forms of therapy have significant side effects, notably bone marrow suppression and secondary infection, and in the case of ASCT a mortality of up to 5%. Progression of the neuropathy is halted by systemic treatments, but recovery is slow and often delayed, sometimes starting only after two years. Once neuropathy is established, recovery is nearly always incomplete. Chemotherapeutics in POEMS syndrome have been associated with worsening of neuropathy (lenalidomide, thalidomide, bortezomib) and therefore non-ASCT treated patients are at further risk of decline. Understanding the pathological mechanism of neuropathy may contribute to the development of nerve-directed therapies in POEMS syndrome. It is conceivable that therapies that have an additional role of neuroprotection or regeneration might prevent any further neurological decline resulting in better outcomes. These might be used in parallel or in combination with therapies directed towards the abnormal plasma cell clone. This could only be achieved through firstly understanding the mechanism leading to neuropathy in POEMS syndrome.

1.7.2 Study hypotheses

1. POEMS syndrome is not as rare as is believed and has a unique set of identifiable clinical features consistent with and beyond those previously described.
2. Sera of patients with POEMS syndrome exhibit a unique cytokine fingerprint which underlies the pathogenic mechanism of disease, including neuropathy.
3. Immunoassays of peripheral nerve proteins will identify peripheral neuropathy from healthy controls and central nervous system disorders.
4. Concentrations of peripheral nerve biomarkers will vary according to disease severity and treatment response.
5. Proteomic analysis of POEMS sera will reveal pathological processes and biomarkers of disease activity.
6. Non-antibody, cytokine mediated neuropathology will be demonstrated from sera of patients with POEMS syndrome in neuronal co-cultures and provide insight to peripheral nerve pathogenesis in POEMS syndrome.

1.7.3 Study aims

1. Recruit a cohort of POEMS syndrome cases and characterise the clinical features, investigation results, treatments and outcomes.
2. Investigate potential novel biomarkers for peripheral nerve damage in POEMS syndrome and related neuropathies.
3. Define the pattern of cytokines and other inflammatory molecules responsible for the pathogenesis of POEMS syndrome.
4. Establish the pathologic mechanisms of these molecules in causing neuropathy.

1.8 Funding and ethical approval

The funding for this study was provided by the Association of British Neurologists and Guarantors of Brain Clinical Research Training Fellowship grant.

Ethical approval for this research study was granted by the National Research Ethics Service (NRES) Committee London (research ethics committee reference 16/LO/1852). All participants provided written informed consent to participate and for their data to be stored in accordance with the Data Protection Act. The study was conducted in accordance with Good Clinical Practice guidelines and the World Medical Association Declaration of Helsinki.

2. Characterising the clinical features of POEMS patients in the UK

2.1 Introduction

The rarity of POEMS syndrome combined with the multitude of clinical characteristics and variable outcomes influenced by increasingly effective therapies render natural history studies complex and laborious. Previous studies have additionally suggested a lack of generalisability across international cohorts, providing rationale towards defining the UK cohort.[7,9,14,15,93] Deep phenotyping to understand more about the natural history of disease may also identify key features of POEMS syndrome which distinguish it from CIDP, assisting in early diagnosis. Developing biomarkers, performing cytokine analysis and cell modelling will require interpretation in conjunction with detailed clinical information to make sense of the data gathered. For example, comparing cytokine profiles to neuropathy severity scores or outcomes following therapy will be fundamental to understanding the relevance of the scientific data. The first step to this research project was to recruit and gather clinical data on the POEMS cohort as the foundation for further scientific research.

The data provided in the chapter below was collated solely for the purpose of this PhD thesis, however sections of the study have been subsequently published in a number of medical journals. [1,3–5,94,95]

2.2 Methods

2.2.1 Study participants and recruitment

A retrospective review of all patients with a diagnosis of POEMS syndrome attending the national POEMS service at University College London Hospital (UCLH), UK from 1998 to 2017 (the start of the research project) was performed, which identified 55 cases. Subsequent to this, various methods were utilised to increase our case numbers for the purpose of developing a strong clinical service and increasing patient recruitment into research, which would involve the collection of clinical information and donation of both serum and plasma on first and subsequent outpatient visits for further testing. Patients who fulfilled the internationally recognised diagnostic criteria for POEMS syndrome, including the two mandatory criteria plus >1 major and >1 minor criteria were included (see table 1).

2.2.1.1 RaDAR scheme

The Rare Diseases Ascertainment and Recruitment is a platform run by the Association of British Neurologists (ABN) to facilitate research recruitment for rare diseases. Following application, all ABN members receive a monthly email listing conditions to be reported. If a member saw a patient with POEMS syndrome, they could record this online which notifies the study team. Following documented patient consent and consultant approval, the ethics committee permits SK to then contact the patient for recruitment into the study, which involves sharing of clinical information +/- plasma and serum collection if practically feasible.

2.2.1.2 Direct referrals

Through publishing POEMS clinical guidelines, producing POEMS information sheets, presenting at conferences and updating the UCLH POEMS service website, we aimed to increase awareness of the POEMS service as the UK's quaternary referral centre. For this reason, we received an increasing number of direct referrals of patients to see in the clinic who would then be invited to participate in the research.

2.2.1.3 VEGF reporting

Since the neuroimmunology department at Queen Square, UCLH is the only United Kingdom Accreditation Service (UKAS) accredited laboratory to carry out VEGF testing in the UK, this would mean most (if not all) UK POEMS patient sera would be tested at our site. We appended a clinical note to the VEGF report stating the significance of a raised VEGF, and provided contact details of our POEMS team for further information and clinical support. When results had been reported, any result over 1000 pg/ml would prompt SK to contact the treating physician directly again to provide clinical support and offer patient review in the UCLH POEMS clinic if deemed to be a likely new case, and subsequently offered to participate in the research study as routine.

2.2.2 Data and sample collection

Clinical features, laboratory, radiological, neurophysiological and histopathological findings were collected, as were treatments and outcome. Patients were reassessed at regular intervals (6 monthly or more dependent on clinical need). Patients' local

physicians or general practitioners were contacted for patients for whom there were incomplete data. Definitions of response to treatment and progression were based on five main domains, and based on previously reported measures. [52,96]

- **Clinical –** Four clinical response categories have been used: Clinical improvement (Ci), clinical progression (Cp), mixed clinical response (Cm) and Clinical stability (Cs). This was based on patient and physician qualitative reports of constitutional symptoms and systemic features.
- **Neuropathy-** defined by clinical examination, neurophysiology, modified Rankin Score [97] and Overall Neuropathy Limitation Score (ONLS). [98] ONLS requires addition of the total Arm scale score (0-5) to the Leg scale score (0-7) yielding a total score of 0-12. Progression defined as worsening of functional ability by an increase of ONLS by ≥1.
- **Haematological –** evaluated according to modified International Myeloma Working Group criteria. [99]
    - Complete response (H-CR): negative bone marrow (BM), negative immunofixation (IF) of serum and urine.
    - Very good partial response (H-VGPR): 90% reduction in Monoclonal (M)-protein or only IF positive as long as M-protein at least 0.5g/dL at baseline.
    - Partial response (H-PR): 50% reduction in M-protein or IF positive as long as baseline M-protein was at least 1.0 g/dL.
    - No response (H-NR): not fulfilling the above.

  Progression defined by re-emergence of serum/urine M-protein if undetectable or increase by 25% from lowest post treatment value.

- **Serum Vascular Endothelial Growth Factor (sVEGF) Biochemical -**

  The upper limit of the normal range (ULN) was determined at 771 pg/mL. Responses were defined as below.

  - Complete response (VEGF-CR): normalisation of levels.
  - Partial response (VEGF-PR): >50% reduction of sVEGF if baseline over 2000 pg/ml.
  - No response (VEGF-NR): doesn't fulfil the above.
  - Not evaluable (VEGF-NE): initial sVEGF not raised/not taken.

  Progression defined by persistent (≥2 recordings) sVEGF elevation ≥771 pg/ml from a previously normal result. If lowest post treatment value was ≥771 pg/ml (i.e. not normalised), a persistent rise in sVEGF of >50% from lowest post treatment value was defined as progression.

- **FDG-PET (radiological) response**

  - Complete response (R-CR): disappearance of Standardised Uptake Units (SuV) avidity.
  - Partial response (R-PR): >50% reduction of SuV avidity of lesions.
  - No response (R-NR): doesn't fulfil the above
  - Not evaluable (R-NE): initial PET scan does not show avidity.

  Progression defined by increase in size or avidity of lesion, or new plasmacytomas.

2.2.3 Central nervous system features of POEMS syndrome

To comprehensively study central nervous system features of POEMS syndrome, a separate methodology was adopted but using the same cohort data. Retrospective review of all brain MRIs and spine MRIs of patients with POEMS syndrome were compared to an age and sex-matched control group of brain and spine MRIs from patients with CIDP (numbers based on availability). Images were interpreted by two consultant neuroradiologists blinded to the diagnosis, with particular attention to vascular and dural/meningeal pathology which had been previously reported in POEMS syndrome but in small cohorts. Meningeal thickness was measured at the point of maximal thickness with >1mm taken as abnormal. The presence of small vessel disease was determined by bilateral patchy of diffuse white matter change, but was not quantified formally. No patient had received treatment for POEMS at the time of the MRI. Clinical indication for MRI imaging was varied but mostly related to focal CNS symptoms or signs or for non-specific symptoms such as headache. Spinal MRI was more routine for work up of demyelinating polyneuropathy. No patients received lumbar puncture within 3 months prior to MRI, but may have had one prior to that.

2.2.4 'The cost of misdiagnosis in POEMS syndrome' economic analysis

This study also utilised data from the POEMS cohort in order to investigate the following:

1. The economic costs associated with misdiagnosing POEMS syndrome patients with CIDP
2. The incremental cost-effectiveness ratio of a new POEMS diagnostic pathway

The economic costs analysis focused on comparing the cost of patients directly diagnosed with POEMS syndrome to patients diagnosed with POEMS subsequent to an incorrect CIDP diagnosis. For each activity leading up to a confirmed POEMS syndrome diagnosis (Figure 2.1), we estimated the quantity of resources used and multiplied these by their respective unit costs. A list of all costing inputs used is included in appendix 1 which presents all inputs and assumptions in this analysis.

As detailed IVIG treatment data including number of treatments and IVIG quantity prescribed were only available for a sub-set of patients (n=26), we used information from the National Immunoglobulin Database to estimate the average cost of IVIG treatment-per-patient, and combined this with unit costs of plasma exchange and corticosteroids to estimate the total costs associated with an incorrect CIDP diagnosis for each patient. By multiplying average cost-per-POEMS syndrome patient misdiagnosed with CIDP by the number of misdiagnosed patients, we estimated the total cost associated with CIDP misdiagnoses across our cohort.

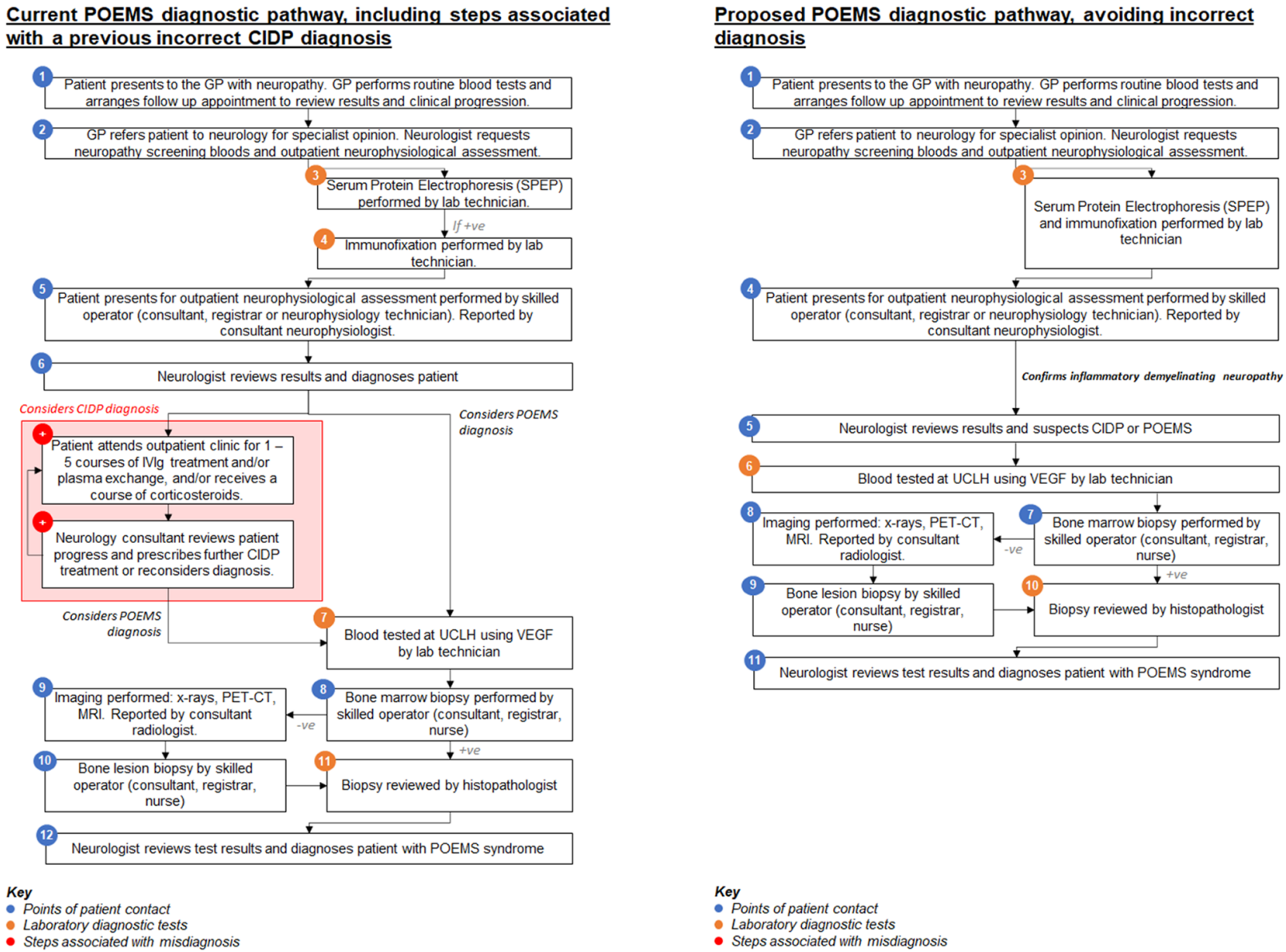
We used decision analytical modelling to compare the cost-effectiveness of the current diagnostic algorithm when investigating a patient with acquired demyelinating neuropathy (standard of care, SOC) with a new diagnostic pathway which includes VEGF testing and mandatory immunofixation which we hypothesised would more accurately identify POEMS syndrome cases from CIDP resulting in lower overall costs. The analytical model is detailed in figure 2.1:

1. Current Standard of care (SOC): SPEP; if positive, immunofixation
2. Intervention: SPEP, and immunofixation. VEGF testing in electrophysiologically confirmed acquired demyelinating polyneuropathy.

We modelled an incidence cohort of 3,635 patients with an inflammatory polyneuropathy as the study population, which we estimated to approximate the annual number of patients referred by a GP to neuromuscular clinics with any inflammatory polyneuropathy in the UK.[100] This estimate was based on the demographically similar population and healthcare system of the Netherlands as the nearest to the UK (appendix 1.4). Patients transitioned through the decision tree according to test accuracy, misdiagnosis and treatment rates (appendix 1.2). We used a time horizon from presentation with polyneuropathy symptoms, until a confirmed, correct diagnosis. Input data and sources are described in appendix 1.3.

Our model estimated the cost associated with each diagnostic pathway. We evaluated cost-effectiveness using the incremental cost-effectiveness ratio (ICER); i.e. the added cost per additional correct POEMS syndrome diagnosis. We carried out one-way deterministic sensitivity analyses (DSA) and probabilistic sensitivity analysis (PSA), as detailed in appendix 1.3.

Figure 2.1: Current POEMS diagnostic pathway (left) vs proposed diagnostic pathway (right)



2.2.5 Designing and utilisation of the Redcap Database

POEMS patient data was designed to be input into Redcap for storage and analysis. This is a secure web application for building and managing online databases for research. The benefits of such an application are that any (approved) clinical user has the information available to them to update or analyse for their own research uses. I used Redcap to design a user-friendly database for collecting cross sectional and longitudinal clinical information for patients with acquired neuromuscular disease at NHNN. Standardised human phenotype ontology (HPO) terms were used to standardise inputs allowing for simpler data retrieval and grouping at a later date. The aim was for the key data entry to be applicable to any neuromuscular patient, but then also to include options to document disease specific data in customisable forms which could be added to the standard skeleton. An example of this is demonstrated in figure 2.2a. The data from Redcap can then be exported to excel for data analysis.

Figure 2.2a: Redcap database flowchart demonstrating generic forms (in green) and automatically linked customised POEMS specific form (blue).

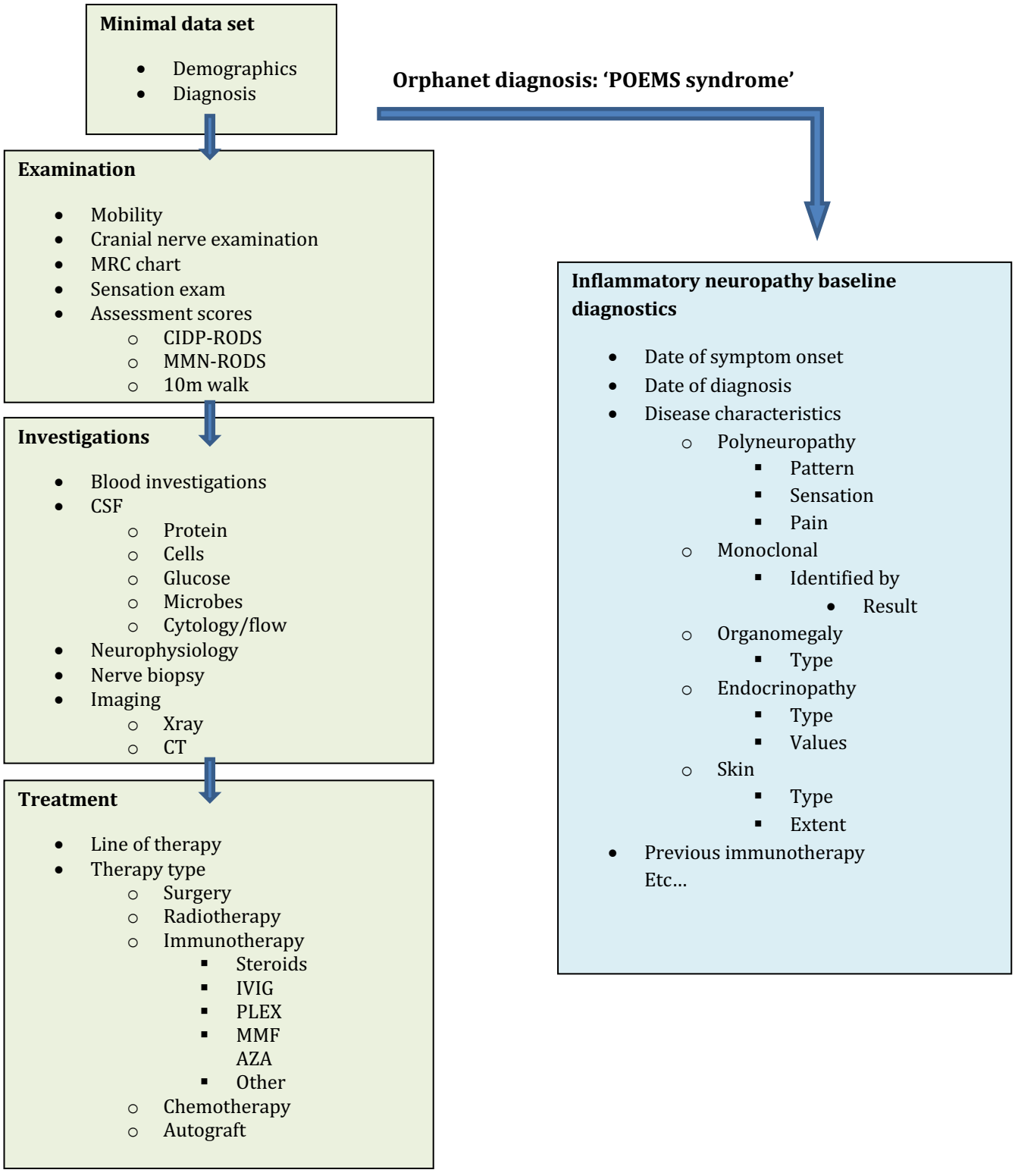


Figure 2.2b: Redcap database demonstrating data collection instruments across time/'events' to collect longitudinal data

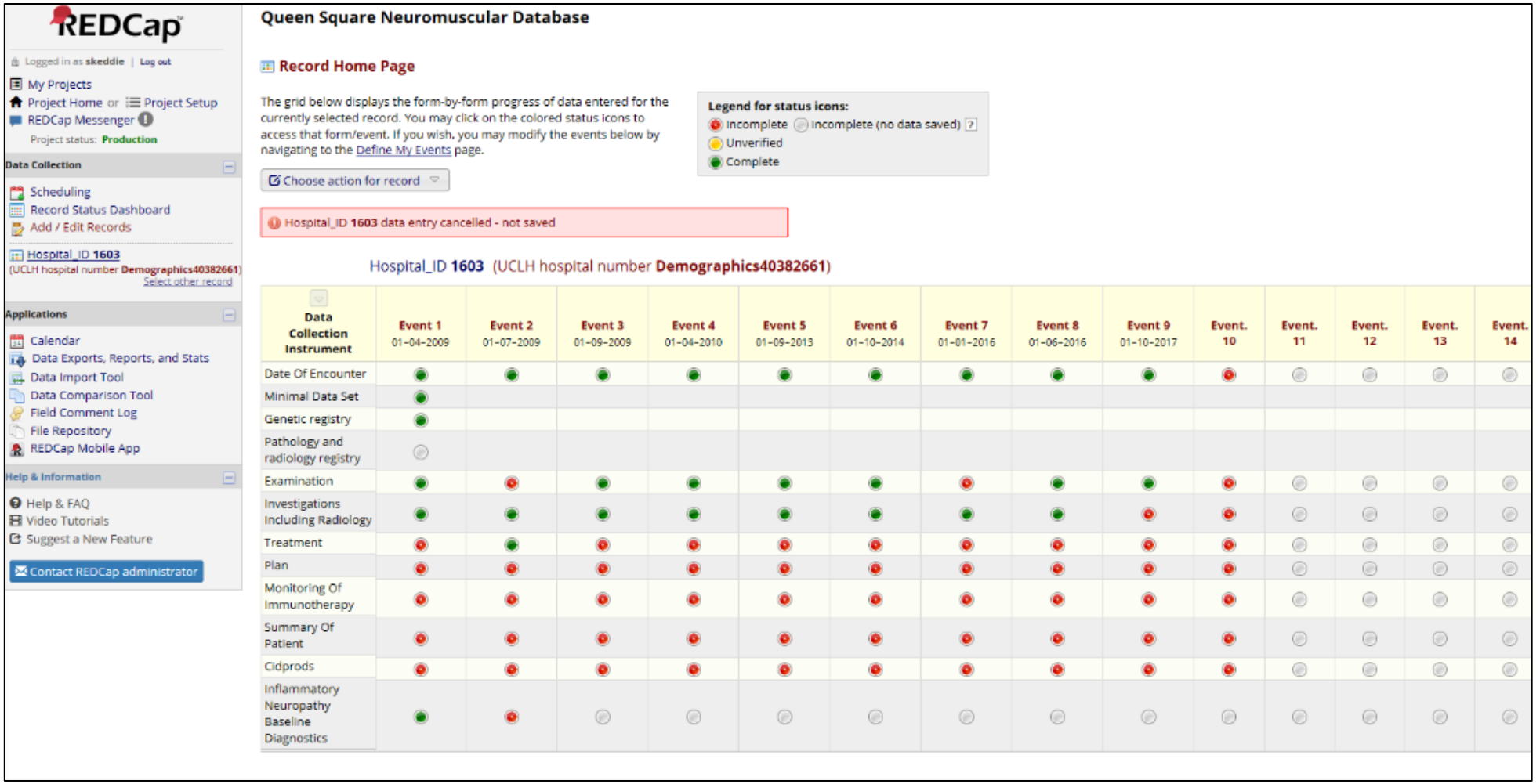
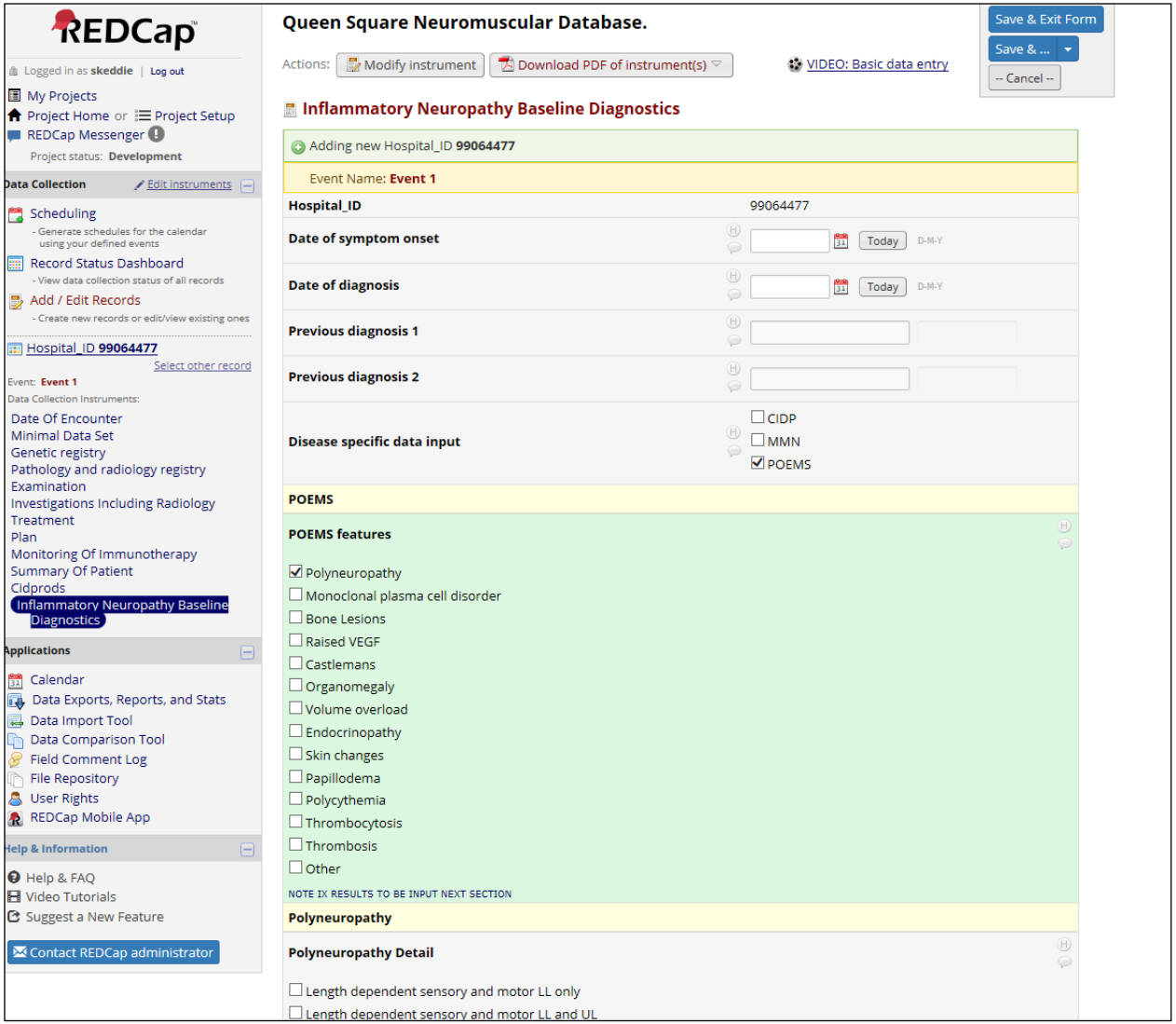


Figure 2.2c: Redcap database displaying 'Inflammatory Neuropathy Baseline Diagnostics' with explodable features specific to POEMS syndrome once that diagnosis has been selected



2.2.6 The neuromuscular neuroimmunology biobank

Patients recruited into the POEMS study consent for 2 serum and 2 EDTA blood samples to be collected during any routine VEGF collection. Samples are then sent to the laboratory at the Neuroimmunology and CSF laboratory, Institute of Neurology and centrifuged at 3000rpm for five minutes, aliquoted into 1.5ml aliquots, labelled with a unique identifier and stored at -80 ^{o}C. Sample ID, location (box number, grid number) and disease was documented on a protected excel database stored on the UCLH secure computer system. Samples were stored for cytokine analysis, biomarker discovery, neurofilament light testing and use in HiPSC cell models. Samples collected for clinical purposes (such as VEGF, ganglioside antibody and serum oligoclonal band testing) are routinely held for five years within the neuroimmunology laboratory and were also available for use under our ethics. This was useful for collecting pre-treatment samples of patients who presented prior to the initiation of this research project but subsequently consented to be involved.

2.2.7 Statistical methods

Baseline characteristics were described using medians and range for non-parametric continuous data, and means and standard deviations for parametric. Wilcoxon signed rank test was performed to compare pre- and post-treatment sVEGF levels. Kaplan-Meier methods were used to plot progression free and overall survival by time. Survival was calculated from time of diagnosis. Progression free survival was defined from the time of treatment to documented progression of disease. Patients who had not reached the endpoint of death or progression at the time of analysis were censored.

Risk factor identification was performed by comparing log-rank univariate analysis of potential risk factors to the outcome of death or progression. Risk factors are listed below:

- Demographics and disease characteristics
    - Age, sex, time to diagnosis, oedema, cardiac failure, pulmonary HTN, restrictive lung disease, obstructive lung disease
- Investigation findings
    - Low albumin, low GFR, presence of paraprotein, Immunoglobulin type, abnormal SFLC, difference in free light chain >10, plasma cell % on biopsy
- Treatments and outcome
    - First line treatment type (radiotherapy, cyclophosphamide, lenalidomide, ASCT), haematological non-response, VEGF non-response

Continuous variables were transformed into categorical variables based on routine cut offs (where available), for example pulmonary hypertension was defined by pulmonary artery systolic pressure >50mmHg. Variables with strong significance ($p<0.01$) were included in a multivariate model. Factors remaining significant in the multivariate model were used to build a risk score for poor outcome (death or progression). The variable with the lowest hazard ratio (HR) was assigned a value of 1, and other variables assigned a score relative to their HR. Risk scores were plotted on Kaplan Meier curves and similar trends combined to identify the optimal number of subgroups.

Statistical analyses and data presentation were performed using IBM SPSS 26 and Graphpad prism 8.0.

2.3 Results

2.3.1 Demographics

A total of 100 patients were included, of which follow-up data were available for 97. Median follow up was 59 months (range 1 to 252 months). Patients were typically male (68%) with a median age of 56. These ethnicity data broadly reflected that of London and the rest of the UK.[101] Sixty three percent of patients were initially diagnosed with another disorder, and median time to diagnosis was of 14 months. The majority of patients presented with six or more POEMS clinical features. Table 2.1 describes these data in more detail.

Table 2.1: Demographic data of 100 POEMS patients

Characteristic	N=100
Median age, y (range)	56 (31-84)
Male sex	68
Ethnicity	
White British	62
White other	16
Black African	11
Black Caribbean	3
Asian Pakistani	2
Asian Indian	4
Chinese	1
Not specified	1
Diagnosis prior to POEMS syndrome	
CIDP	55
GBS	5
MGUS	1
B12 deficient neuropathy	1
Scleroderma	1
Number of POEMS features at diagnosis	
3 features	1
4 features	3
5 features	9
6 features	25
7 features	22
8 features	22
9 features	10
>10 features	8

2.3.2 Characteristic features of POEMS syndrome

2.3.2.1 Polyneuropathy

Ninety-six percent of patients had evidence of polyneuropathy on examination and neurophysiological testing. Two patients without evidence of polyneuropathy had the Castleman variant POEMS syndrome, in which the neuropathy is typically mild. [102] The majority (90%) of patients presented with a sensorimotor neuropathy, with 5% presenting with pure sensory symptoms, and 1% pure motor. Three of the five patients with pure sensory neuropathy had the Castleman variant of POEMS. A definite length dependent, symmetrical progression in sensory and motor symptoms was observed in 93% of those with neuropathy. No patients presented with polyradiculoneuropathy with early proximal involvement.

On presentation, sensory symptoms were varied with numbness most commonly to the foot only in 14%, 'short sock' distribution in 13% and the knees in 25%. In the upper limbs, numbness was most commonly found at presentation to the wrists in 26%. Sensory examination was varied, but most commonly vibration was reduced to the elbows and the anterior superior iliac spine, pin prick to the mid forearm and the knees, and joint position sensation normal in the upper limbs and absent at the hallux. Pain was a commonly reported symptom, with 75% of patients complaining of neuropathic pain, often described as a bilateral cramping, tight sensation in the calf, but not specifically cramp.

By the time POEMS was diagnosed, 9% of patients were bedbound requiring hoists to transfer and 27% were wheelchair users. Of those still ambulant, 14% were already using a frame, 26% using one or two sticks and 23% unaided or using orthotics (see figure 1). The median modified Rankin Scale (mRS) was 3 and the median ONLS was 6 at presentation. In contrast the median ONLS for the Castleman variant POEMS patients (n=14) was 2.

Neurophysiological data were available and collated for 96 patients. Findings were broadly homogenous, with reports of a sensory (83%) and motor (87%) neuropathy with conduction velocity slowing (85%) and secondary axonal loss (72%). Four studies were reported as normal, two of which were in Castleman variant patients without clinical evidence of neuropathy. Only two studies reported conduction block and none temporal dispersion.

When a lumbar puncture was performed (65%), cerebrospinal fluid (CSF) protein was abnormally raised in all samples, the median value being 1.25g/L (range 0.6-9 g/L, normal <0.4 g/L).

A sensory nerve biopsy was performed in 17 cases. All cases were reported as having loss of large and small myelinated fibres (ranging from mild to severe fibre loss). Review of 12 biopsies showed frequent active axonal degeneration in seven, occasional in three and absent in two. Widespread epineural neovascularisation with haemosiderin deposits was seen in one biopsy, with focal neovascularisation evident in

a further three biopsies. Epineural perivascular lymphocytes were mildly increased in four, scarce in six and absent in two biopsies. Endoneurial T lymphocytes were frequent (up to 10 per transverse section) in three biopsies and rare to absent in the other nine biopsies. Teased fibres were available for four biopsies and showed frequent segmental demyelination in a single case with no evidence of segmental demyelination in the other three cases. Electron microscopy was available for review for 10 cases and showed un-compacted myelin in five. Onion bulbs, a feature of chronic demyelinating / re-myelinating process, were not seen in any of the biopsies.

2.3.2.2 Central nervous system features

Central nervous system involvement had been reported previously in case reports but was not considered by other authors to be a key feature.

Nine patients experienced stroke, seven of which were ischaemic and two subarachnoid haemorrhages. Ischaemic stroke was the presenting feature in three patients before other systemic features were discovered and the diagnosis of POEMS made. Of the ischaemic strokes, there were three middle cerebral artery infarcts, one lacunar, and three in the posterior circulation. Four of the nine (44%) patients had concomitant thrombocytosis, and one polycythaemia. Three of these four with thrombocytosis additionally experienced venous thromboembolism.

Forty-one MRI brain scans from POEMS patients were compared to 19 of patients with CIDP. MRI scans from POEMS patients demonstrated smooth diffuse meningeal thickening of the cerebral convexities and falx in 29/41 (71%). Maximal dural thickness varied with mean 2.1mm (range 1-5mm) with the area of maximal thickness most often being in the frontal region. Four cases were found to have meningeal collections, of which imaging features were consistent with free fluid. None of the 19 CIDP patients with brain MRI had meningeal thickening ($p<0.0001$ vs POEMS).

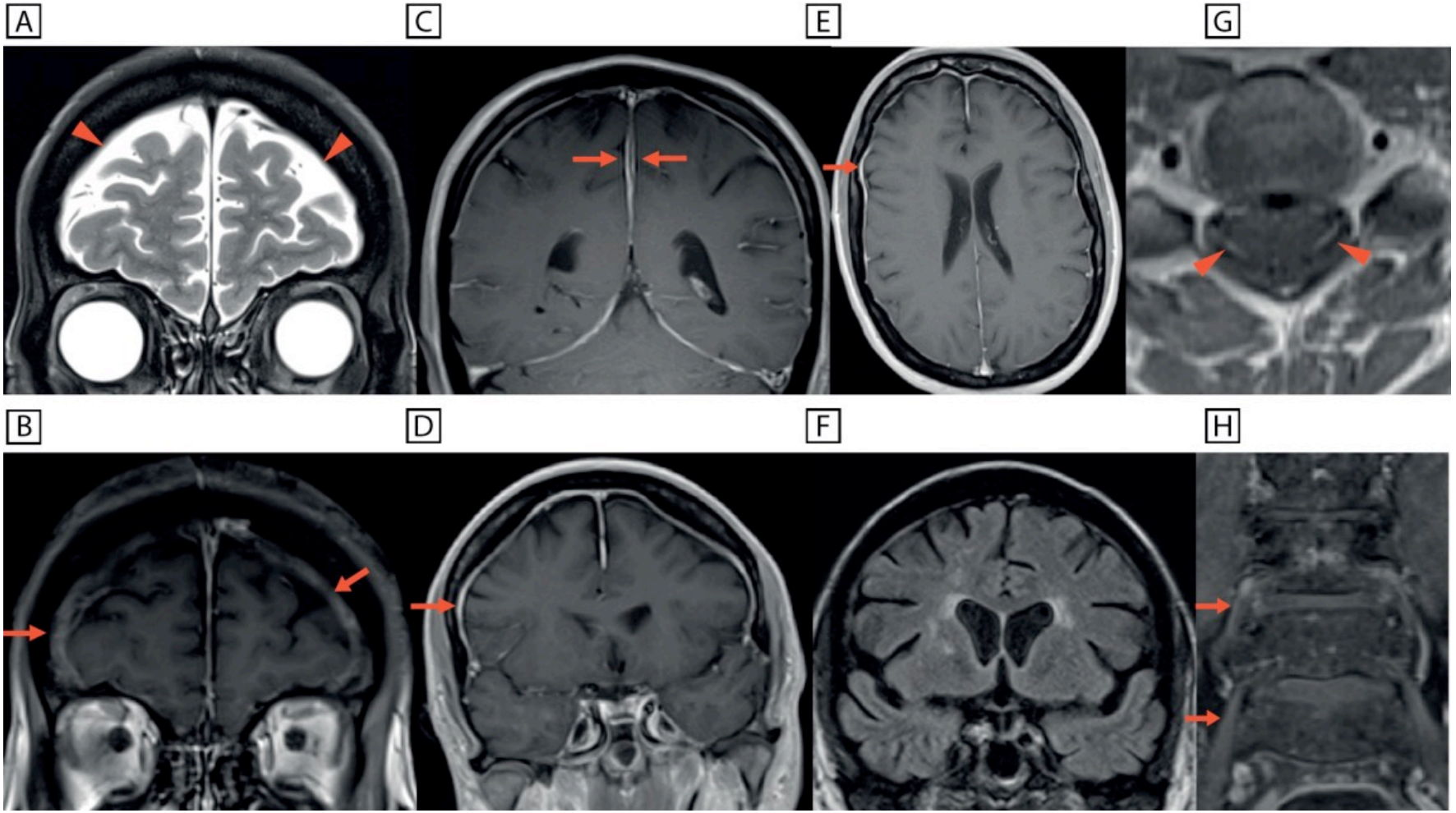
White matter abnormalities consistent with small vessel disease were found in17/41 POEMS patients (41%). Four of these (10%) were established infarcts. Comparatively, eight of 19 (42%) CIDP patients had white matter abnormalities of similar distribution ($p=0.85$ vs POEMS). Although not quantified, the extent of small vessel disease did not appear to differ between the two groups.

Neither meningeal thickness nor vascular abnormalities were correlated with VEGF level ($r=0.05$, $p=0.78$: $r=0.02$, $p=0.89$ respectively) or age ($r=0.33$, $p=0.06$; $r=0.18$, $p=0.33$). Although meningeal thickness was not correlated to symptom duration ($r=0,18$, $p=0.26$), a significant association was identified between CNS vascular abnormalities and symptom duration ($r=0.50$, $p=0.002$).

17 of 29 POEMS (44%) patients were found to have thickening and enhancement of the nerve roots. This involved the brachial and lumbrosacral plexus in 15 cases and the brachial plexus only in the remaining two. No spinal cord abnormalities were present. Nine of 26 (34.6%) CIDP patients had thickening and enhancement of nerve roots

(p=0.06 vs POEMS) involving the brachial plexus in seven, lumbosacral plexus in one and both in the remaining patient.

Figure 2.3: CNS findings in POEMS syndrome



A *Coronal T2w image: widening of subdural planes over bifrontal convexities.* ***B*** *Coronal post-gadolinium T1w equivalent: thick enhancing dura in this area.* ***C*** *Post-gadolinium T1w coronal image: two opposing layers of enhancing dura.* ***D*** *Postgadolinium T1w image: diffuse enhancement of intracranial pachymeninges.* ***E*** *Post-gadolinium T1w image: focal enhancement of intracranial pachymeninges.* ***F*** *Coronal FLAIR: moderate degree of small vessel disease.* ***G*** *Post-gadolinium T1w image of the cervical spine level C5 showing intradural leptomeningeal enhancement of cervical nerve roots.* ***H*** *Thickened and enhancing roots/proximal lumbar plexus on post-gadolinium T1w coronal image.*

2.3.2.3 Monoclonal plasma cell dyscrasia

Of the 100 patients, 55% were found to have a paraprotein on serum protein electrophoresis (SPE). The median paraprotein level was 4 g/dL (range undetectable-17 dL). Serum immunofixation (IF) detected an abnormality in 78% of which 34 (44%) were monoclonal IgG, 42 (53%) were monoclonal IgA, and two were biclonal (3%). Seventy-seven of 78 (98%) were lambda light chain restricted, and only one patient had a kappa paraprotein. Had immunofixation not been performed, a monoclonal protein would have been missed in 23 patients.

Serum free light chains (SFLC) were abnormal in 65 of 82 cases (79%) performed at presentation. The kappa:lambda SFLC ratio was normal on 65 occasions (78%), high in only 14 and low in four illustrating the low detection ability for this test. SFLC demonstrated both raised kappa and lambda in 41 cases (50%), raised kappa and normal lambda in 16 (20%), and normal kappa and raised lambda in only eight (10%). When the kappa light chain was raised with normal lambda, the kappa light chains were only minimally raised above the upper limit of normal, with a median value of 28.5 mg/L (upper limit 19.4 mg/L). In such cases, lambda light chains were often concurrently raised but under the upper limit of normal, with a median level of 17.3 mg/L (upper limit 26.3 mg/L), resulting in a median ratio of 1.8. The most likely abnormal result is both raised kappa and lambda SFLCs, resulting in a normal kappa:lambda ratio.

Urinary Bence Jones protein (BJP) was detectable in 15 cases. In five cases, BJP was detectable where the SPE and IF were negative on initial testing, and therefore would otherwise have been missed during routine non-invasive investigation.

Twenty-one cases had both negative serum protein electrophoresis and immunofixation. A monoclonal plasma cell disorder was confirmed in 11 of these cases through bone marrow biopsy and ten targeted plasmacytoma biopsy.

2.3.2.4 Bone marrow histopathology

Bone marrow aspirate and trephine was performed in 86 of 100 cases. Thirty one of 86 (36%) were reported as normal, and 55/86 (64%) were reported as having features of a plasma cell neoplasm with abnormal CD138 plasma cells and lambda light chain restriction in 30/55 (54%). A clonal population of between 1-5% was most commonly reported on bone marrow histology (42%). The most frequent histological description was of megakaryocyte hyperplasia.

Patients who did not undergo bone marrow biopsy had histopathological confirmation of plasmacytoma through targeted bone lesion biopsy. Targeted bone lesion biopsy was performed in 42% cases. Sites for biopsy included the pelvis (n=27), spine (n=15), humerus (n=2), ribs, sternum and skull (n=1). Thirty-eight of 42 were abnormal (90%), with median plasma cell proportion of 10%.

2.3.2.5 Castleman disease

Castleman disease (CD)is a rare lymphoproliferative disorder of angiofollicular lymph node hyperplasia associated with POEMS syndrome. Classification of CD is based upon the degree of lymph node involvement, including unicentric CD limited to one lymph node, and multicentric CD which involves multiple and is often associated with systemic inflammatory symptoms and organ dysfunction due to cytokine dysregulation.[103] Forty-two patients were found to have lymph node enlargement on imaging, 18 of which underwent a targeted lymph node biopsy and typical CD histology was found in 14/18 (77%). 24 of 48 did not undergo lymph node biopsy because the additional discovery of CD histology in patients with a secure POEMS diagnosis was not deemed clinically relevant and thus biopsy avoided.

2.3.2.6 Endocrinopathy

Fifty-nine patients had detailed endocrine follow up. Fifty-four patients (91.5%) had an endocrinopathy at some point during follow-up (25.4% with one endocrine abnormality, 23.7% with two, 28.8% with three, 8.5% with four, 3.4% with five, and 1.7% with six abnormalities).

Hypogonadism

Thirty-nine (68.4%) presented with hypogonadism by the end of follow up. Median time to hypogonadism was 3 years. Of the 29 men with hypogonadism, 15 (52%) were treated with testosterone. Seven were treated with injections and eight with gel. Six

treated developed polycythaemia in comparison to three who did not receive testosterone therapy (p=0.024).

Hyperprolactinaemia

Thirty-three (55.9%) developed hyperprolactinemia, although 6 were already taking therapies which could induce hyperprolactinaemia. Nine underwent MRI brain imaging, eight of which had normal findings. 69.2% with hyperprolactinaemia had hypogonadism. No patient received treatment with a dopamine agonist. In 14 (42.4%) of patients, prolactin levels normalised by the end of follow up.

Thyroid disease

Thirty-two (52%) were diagnosed with hypothyroidism. Hypothyroidism developed after 2.6 years, and resolved in 11 (34%).

Abnormal glucose metabolism

Fourteen (23.8%) presented with abnormal glucose metabolism, eight (13%) with diabetes mellitus and six (10%) with prediabetes. Patients were treated with diet alone in one, oral therapies in four, and insulin in three. Patients developed abnormal glucose tolerance at a median of 3.4 years following diagnosis.

Ten (17%) developed adrenal insufficiency by the end of follow up. Six were primary and four likely secondary from long term steroid use. Median time to develop cortisol deficiency was 3.6 years following diagnosis.

2.3.2.7 Organomegaly and lymphadenopathy

Organomegaly was seen in 63% of cases. Forty-two percent were found to have lymphadenopathy, 31% had splenomegaly and 23% hepatomegaly. Fifty percent of patients with organomegaly had more than one organ involved. There were no other organs reported as being abnormally enlarged or different in appearance.

2.3.2.8 Skin changes

Skin changes were detected in 69/100 (69%) of patients. The commonest abnormality was acrocyanosis (46%) followed by hypertrichosis (25%) and glomerular haemangiomata in 23% patients. Scleroderma-like skin thickening was seen in 14%. Nail changes were noted in 25% patients, the most common description being a leukonychia, and clubbing in 10%.

2.3.2.9 Extravascular volume overload

At presentation, 70% had evidence of extravascular volume overload. Peripheral oedema, pleural effusion and ascites was seen in 66%, 16% and 6% patients respectively. Three patients required recurrent ascitic drainage procedures.

2.3.2.10 Papilloedema

Papilloedema was recorded in 30 patients (30%) with one displaying severe subretinal haemorrhage. Not every patient had formal ophthalmology review with retinal

photographs, and therefore this value may be underreported, but is similar to other reported series. [9] Again, records of resolution of papilloedema were inaccurate but anecdotally, improvement in papilloedema tended to occur in parallel to improvement with other clinical features including reduction in VEGF.

2.3.2.11 Bone lesions

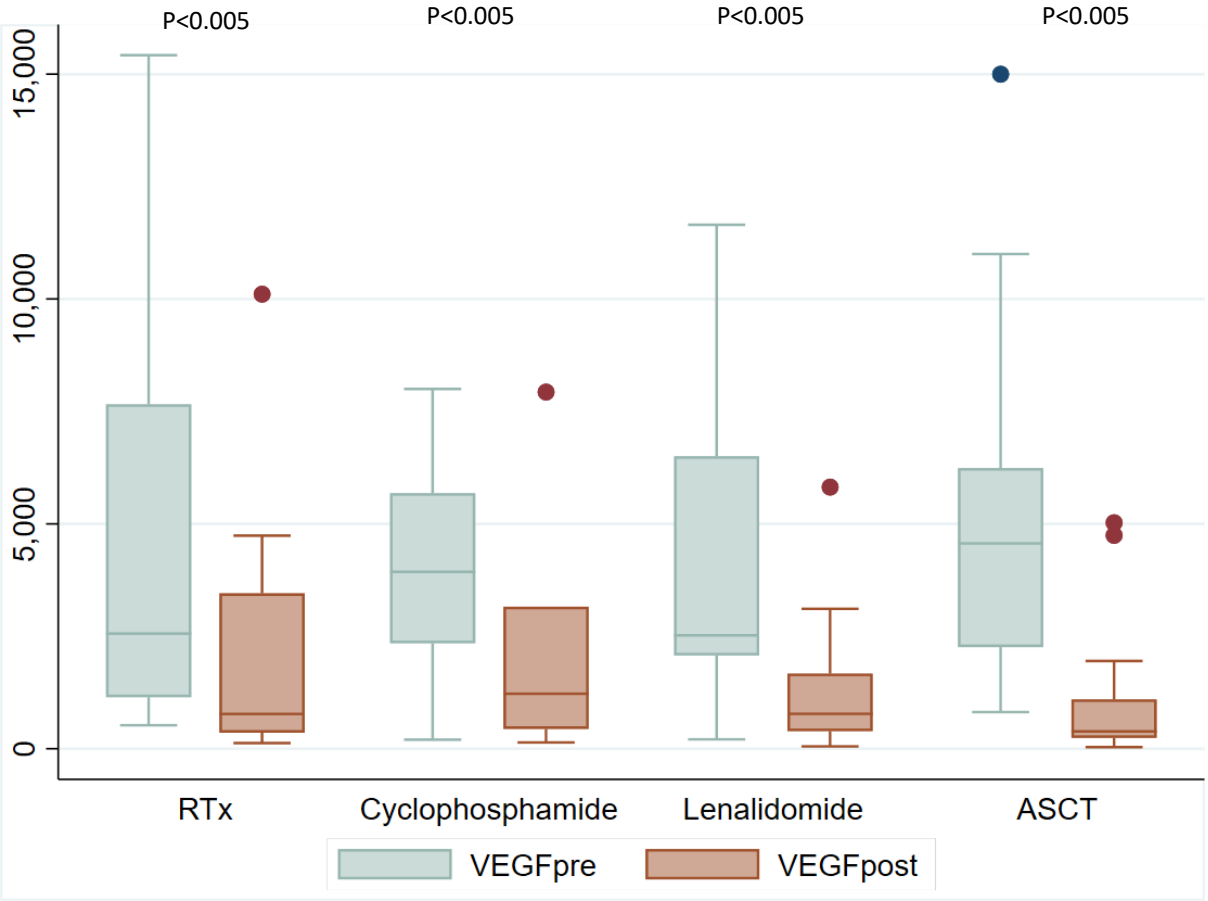
A total of 81% were found to have at least one bone lesion detected on radiographic imaging (skeletal survey, CT, MRI, bone scan, PET-CT). A solitary lesion was detected in 31 cases, two to three lesions in 20 and over three in 30. The majority of lesions were reported as being sclerotic (45), with 12 lytic and 24 were mixed lytic and sclerotic.

2.3.2.12 Vascular endothelial growth factor

Serum (s) VEGF has been routinely available at UCLH since 2009, although some patients prior to this date had sVEGF tested on an ad hoc research basis. Pre-treatment sVEGF was available on 89% patients and was above normal in 84 (94%). Eleven pre-treatment sVEGFs were not available. Median pre-treatment VEGF was 3594 pg/ml, ranging from 200 to 30,101 pg/ml. sVEGF levels taken in the 6 months following systemic therapy demonstrate a significant reduction with a median sVEGF of 685 pg/ml ($z=6.578$, $p<0.0005$). Each treatment modality demonstrated significant reduction of sVEGF post treatment (radiotherapy, autologous stem cell transplantation (ASCT), lenalidomide and cyclophosphamide to $p<0.005$) (see figure 2.3 for details).

Serum VEGF results both before and after treatment were available in 89 cases, demonstrating a biochemical VEGF-CR in 50 (56%), VEGF-PR in 21 (23%) and VEGF-NR in 18 (20%).

Figure 2.4: Serum Vascular Endothelial Growth Factor values pre- and post-treatment



Pre and post treatment levels stratified by treatment options; radiotherapy (RTx), Cyclophosphamide, Lenalidomide or Autologous Stem Cell Transplantation each demonstrated significant reduction following therapy at p<0.005. Dots represent outliers i.e values which were 1.5x the upper quartile.

2.3.2.13 Clotting and thrombocytosis

Thirty-four percent of patients had thrombocytosis and eighteen polycythaemia. All polycythaemic patients were negative for a mutation in the JAK2 or TET2 genes. Eighty-three cases had sufficient information documented to assess for thrombosis. Of the 83, 27 (33%) thrombotic events were recorded. Sixteen were arterial with nine strokes, four myocardial infarctions and three peripheral artery occlusions. Thirteen of these events occurred pre-treatment, and 0 events occurred when the VEGF was <1000 pg/ml. Eleven events were venous, with four deep vein thromboses (DVT), 3 pulmonary emboli (PE), 3 PICC line associated clots and one portal vein thrombosis. Clinical features, haemoglobin, platelet count, VEGF, creatinine or albumin levels did not differ between patients who did and did not develop thrombosis. Most events (22/27) occurred during active disease i.e from development of symptom onset until the initiation of therapy, and five whilst being treated. Twenty thrombotic events occurred in patients not taking thromboprophylaxis. VEGF was noted to be significantly raised in patients leading up to thrombosis, with median VEGF of 2834 pg/ml compared to 6 months following thrombosis at 654 pg/ml, more likely an association of both factors being linked to disease activity rather than any causative relationship.

2.3.3 'The cost of misdiagnosis in POEMS syndrome' economic analysis

Cost of CIDP misdiagnosis

There were 55 misdiagnoses of CIDP in the POEMS cohort (n=100). Misdiagnosed patients received between one and 10 treatments of IVIG (median 3, IQR: 1-5), and a median of 180 grams-per-treatment (IQR: 146–347g). The median Ig-cost per patient

was £7,650 (IQR: £6,216-£14,769) and delivery cost, £12,795 (IQR: £4,265–£21,325). The median total IVIG treatment cost per patient with a CIDP misdiagnosis was £20,984 (IQR: £11,809– £30,349). Misdiagnosed patients (n=55) were compared against patients whom were initially diagnosed with POEMS syndrome (n=45). There was no statistically significant difference between groups in the presenting age, sex, ethnicity or number of clinical features at diagnosis (although these may have been missed upon initial assessment), including the overall neuropathy limitation scale. All patients initially diagnosed as having CIDP were made by a neurologist. Data was lacking regarding whether the diagnosis was made in a specialist neuromuscular unit.

If one assumes patients misdiagnosed with CIDP (n=55) with missing treatment information (n=15) received no treatment, the total costs of CIDP misdiagnosis across our cohort is £808,550 (average cost £14,701). However, if one assumes these patients received treatment in the same proportions as the cohort for which treatment information is known (n=40) the total costs of CIDP misdiagnoses is £1,111,756 with a median cost of £20,214 per patient (IQR: 11,808 –30,348).

Cost-effectiveness analysis

The intervention diagnostic algorithm, in which all patients with acquired demyelinating polyneuropathy were screened with VEGF (including SPEP and immunofixation), would save £107,398 and result in 15.6 additional POEMS syndrome patients directly diagnosed each year across the UK (Table 1). The sensitivity analysis shows that the intervention dominated the SoC across uncertainty values (appendix 1).

Table 2.2: The cost of misdiagnosis in POEMS syndrome economic analysis**.** Taken from Marsh E, Keddie S *et al: Early VEGF testing in inflammatory neuropathy avoids POEMS syndrome misdiagnosis and associated costs. JNNP*

Costing analysis

	Patients with CIDP misdiagnosis	**IVIG**		**Steroids**		**Plasma Exchange**		**Cost**	
Cohort	***n***	***n***	***Cost, £***	***n***	***Cost, £***	***n***	***Cost, £***	***Total, £***	***Av, £***
Conservative*	55	38	797,383[1]	19	289[2]	6	10,879[3]	808,550	14,701
Extrapolated**	55	52[4]	1,096,401[1]	26	397[2]	8.25	14,958[3]	1,111,756	20,214

Cost-effectiveness analysis

	Correct diagnoses	**Total cost, GBP** *(incorrect treatment costs, IFIX + VEGF screening costs)*
Standard of care	12.5	£2,813,462 *(£213,107, £98,007)*[5]
Intervention	28.1	£2,706,064 *(£26,334, £179,584)*
Incremental effect and costs	15.6	-£107,398
Incremental cost effectiveness ratio	Dominates (£6,880 ***saved*** for each correct diagnosis)	

* Assuming patients with no treatment information (n=15) received no treatment

** Assuming patients with missing treatment information received treatment in the same proportions as those for which treatment is known

1. Calculated by multiplying patients receiving IVIG [n] by median IVG treatment cost (£20,984)
2. Calculated by multiplying patients receiving treatment [n] by cost per course (£1,813.12; Supplement I)
3. Calculated by multiplying patients receiving treatment [n] by cost per course (£15.20; Supplement I)
4. ((38 (patients recorded to receive IVIG)/ 40 (patients with treatment information)) x 55
5. Total const includes initial screening, GP appointment, neurology appointment etc as per flow chart figure 2.1

2.3.4 Treatment

Treatments prior to POEMS diagnosis

As previously mentioned, 63/100 cases were misdiagnosed prior to the POEMS diagnosis being made, 55 of which were CIDP and 5 GBS (see table 2.1). Due to the retrospective nature of data collection, treatment may have not always been documented in referral letters and other sources of patient information. However, single or multiple courses of intravenous immunoglobulin (IVIG) were recorded in 40 of patients and glucocorticoids to 21 before the diagnosis of POEMS was established. Ten received immunomodulatory therapies (azathioprine, mycophenolate mofetil, cyclophosphamide, methotrexate and/or rituximab).

POEMS directed therapies

To date, 98 patients have received at least one modality of POEMS directed therapy. Sixty-seven required only one treatment modality, compared to 21 who required a second line, and 10 three or more treatments. In all cases, where systemic treatment was given, this was in conjunction with steroids, usually dexamethasone 80 mg to 160 mg per cycle. See table 2.3 for frequencies of treatment modalities used.

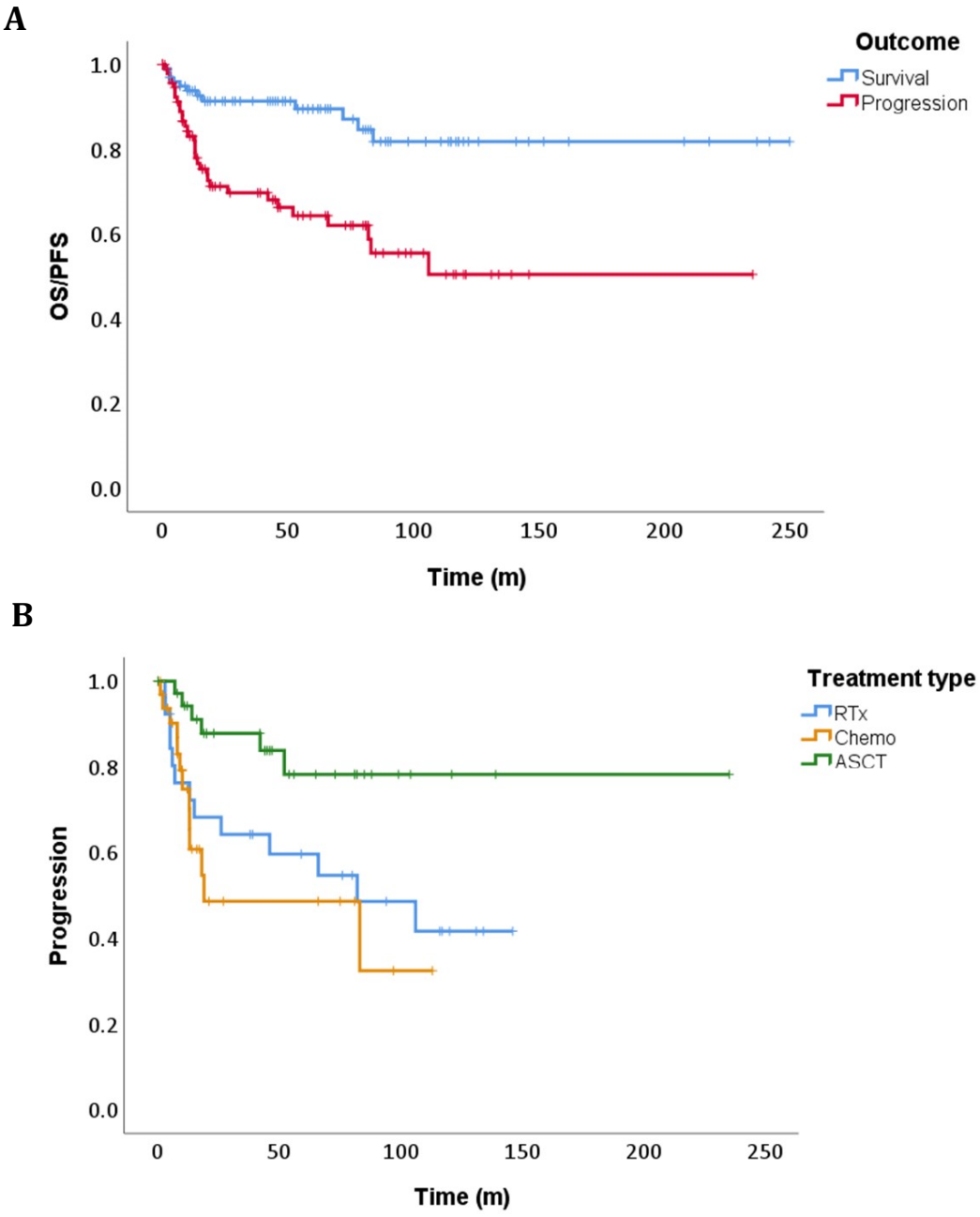
Table 2.3: Treatment modalities used in POEMS syndrome.

Treatment	First line	Second line	Third line	Total Number
Radiotherapy	25			25
Cyclophosphamide	8	6		14
Melphalan	2	1	2	5
Bortezomib	3	2		5
Lenalidomide	23	9	3	35
Autologous SCT	32	15	5	52

2.3.5 Outcomes and survival

The median follow-up of this cohort was 59 months (range 1-230 months) and 7279 person months in total analysis time. The median overall survival (OS) was not reached (figure 7a). Five-year OS was 90% (95% CI 83-96%) and 10 year 82% (95% CI 71%-92%). Twelve patients from the 100 studied have died. Causes of death have been due to POEMS syndrome in seven, cardiopulmonary failure in three, myelodysplastic syndrome in one and multi-organ failure due to sepsis in one. Five-year Progression free survival (PFS) was 65% (95% CI 53%-76%) and ten-year PFS was 53% (95% CI 37%-68%), with progression of any sort occurring in a total of 32 patients (see figure 2.4). Pairwise log-rank comparisons demonstrate a statistically significant prolongation of PFS for patients treated with ASCT vs chemotherapy, X^2 (1) = 9.58, p= .002, and ASCT vs radiotherapy, X^2 (1)= 5.27, p=0.022.

Figure 2.5: Kaplan Meier overall survival and treatment outcomes.

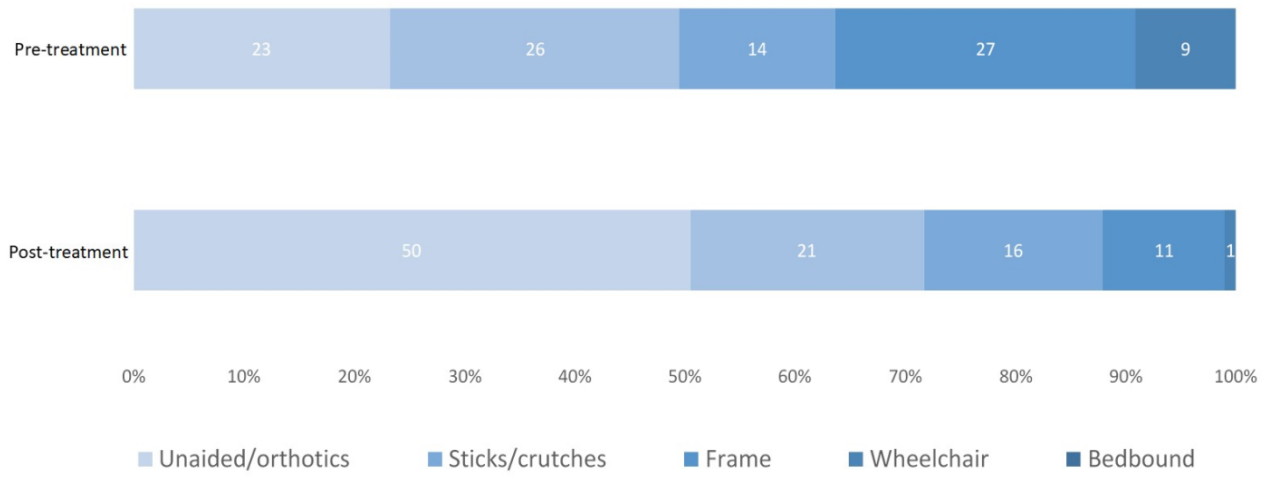


Kaplan Meier survival estimate curves demonstrating (A) = overall survival (OS) and progression free survival (PFS). (B) = progression free survival by treatment types of radiotherapy (RTx) vs chemotherapy vs autologous stem cell transplantation (ASCT).

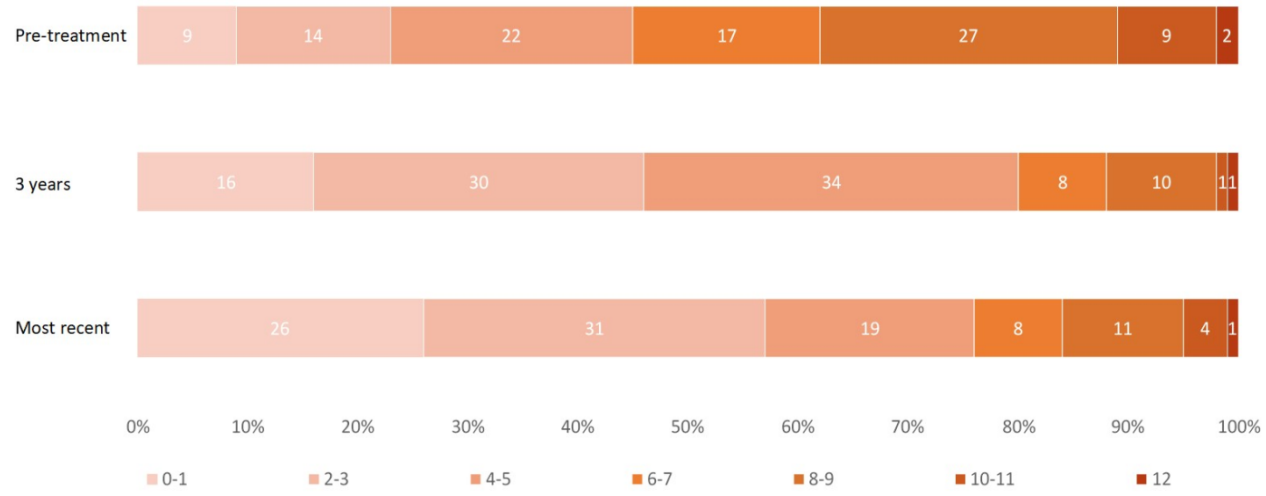
Thirty-five percent of patients were either wheelchair or bedbound prior to receiving treatment, compared to 11% post treatment. Overall median pre-treatment ONLS score was six, which improved to four by three years and three at the most recent patient follow up recorded, demonstrating significant ongoing improvement following treatment ($p<0.05$ - see figure 8). Patients in whom the diagnosis of POEMS was made in less than six months from symptom onset had significantly lower ONLS scores at four, compared to those diagnosed after six months at six ($p<0.05$) suggesting that delay to diagnosis influences neuropathy severity and possibly influences overall long-term disability.

Figure 2.6: Level of mobility and Overall Neuropathy Limitation Score pre- and post-treatment.

A



B



(A)=Mobility level pre-treatment and on most recent follow up (as percentage). (B)= ONLS taken pre-treatment, at three years post treatment (if applicable) and on most recent follow up (as percentage) demonstrating sustained and improving neurological function several years following therapy.

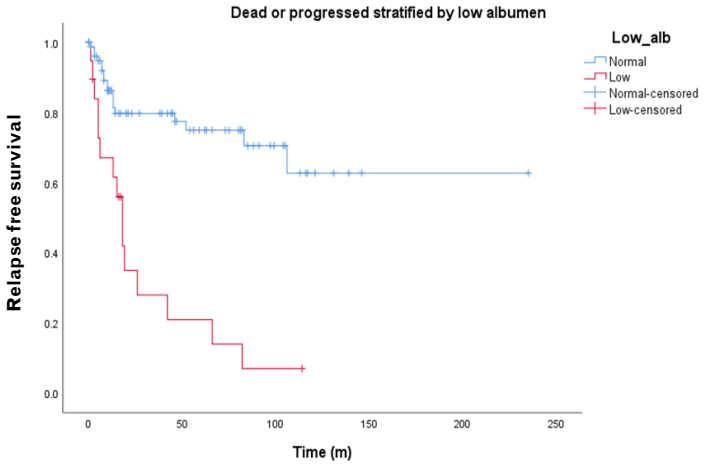
2.3.6 Death and Progression risk factors

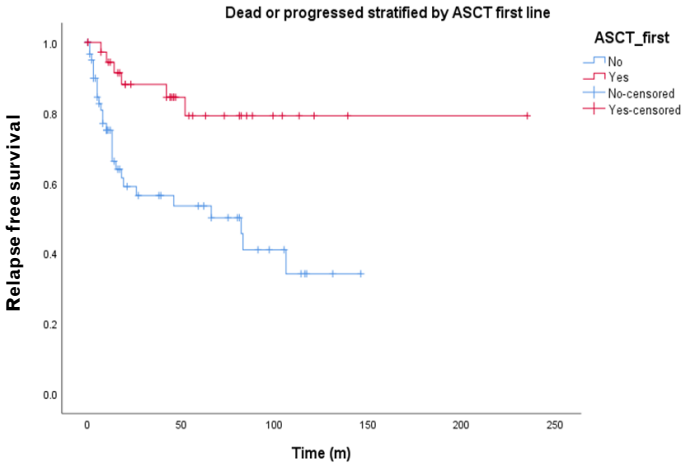
Variables from the univariate analysis which demonstrated strong significance and thus selected for multivariate analysis were haematological non-response, VEGF non-response, non-ASCT therapy and low albumin (see figure 2.7). Variates remaining significant in this model were haematological non-response (HR 4.8, 95% confidence interval (CI) 2.0-11.7, P=0.000), VEGF non-response (HR 2.7, 95% CI 1.0-7.1, P=0.033) and non-ASCT therapy (at any stage of the patients' treatment) (HR 4.5, 95% CI 1.6-12.4, P=0.003). Low albumin lost significance in the model (HR 1.6, 95% CI 0.7-3.8) and was therefore not included in further analysis. Risk scores were ascertained relative to respective HRs as follows; VEGF-non-response, score 1; Haematological non-response, score 2; non-ASCT, score 2, with a total risk score of five.

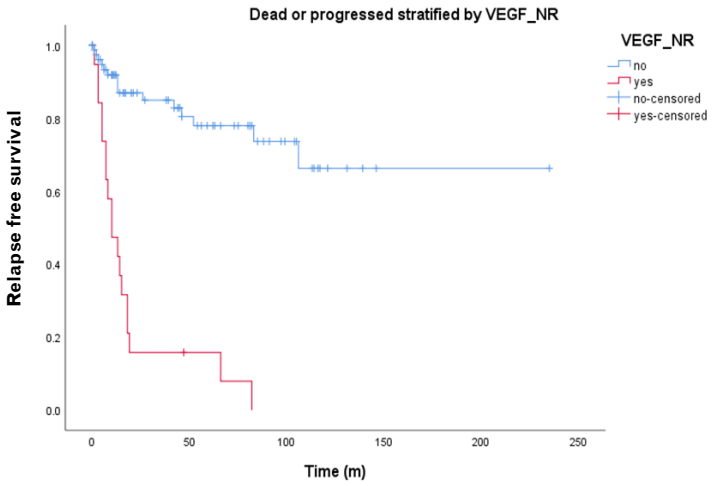
Survival curves were compared for patients with all calculated risk scores and two distinct sub-groups created; low risk, score 0-2; high risk, score 3-5. Pairwise log rank comparisons demonstrated a statistically significant difference in progression or death outcomes between high and low risk groups, $X^2 = 63.8$, p=0.000 (Figure 2.7).

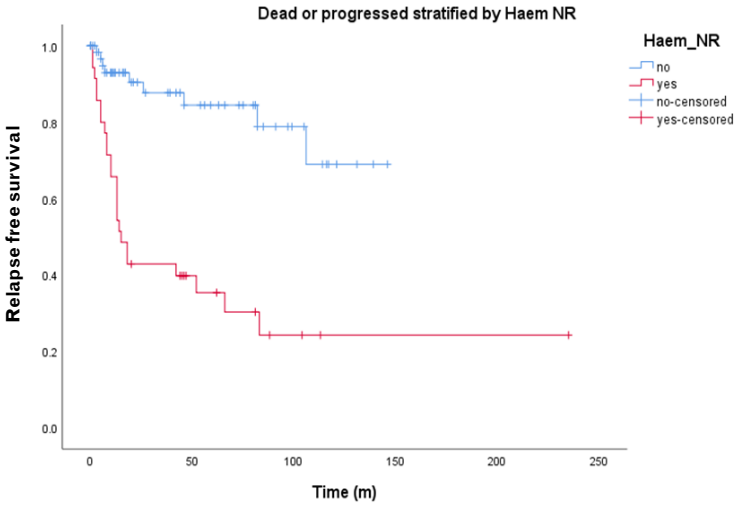
Figure 2.7: (A) = Kaplan Meier curves demonstrating the impact of statistically significant univariate risk factors on risk of progression and death in POEMS syndrome. (B) = Overall survival and progression free survival outcome stratified by risk score.

A

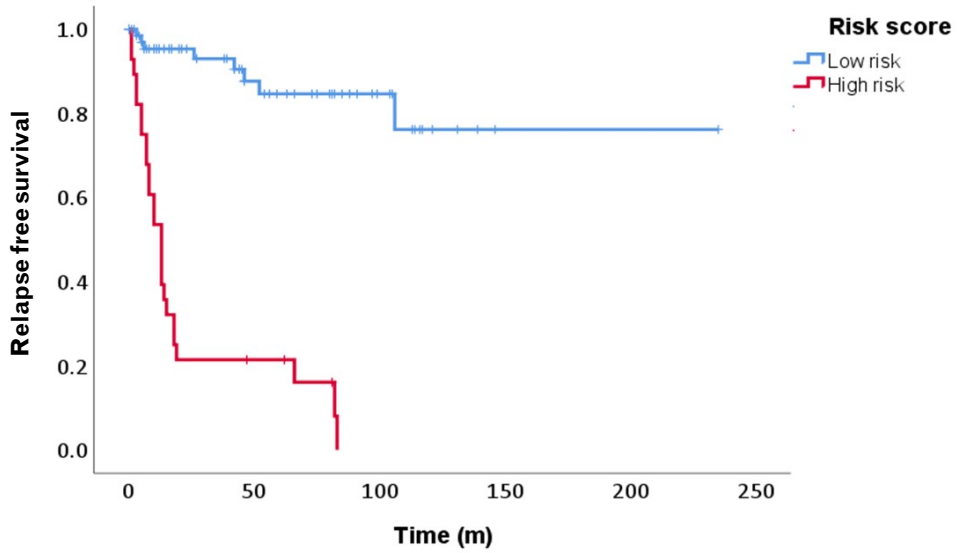








B



2.4 Discussion

This retrospective longitudinal cohort study describes the clinical features, investigational findings, treatments and outcomes of the largest population of POEMS cases in Europe. POEMS syndrome is a rare, multi-system, complex disease which often results in disability, multiple comorbidities and occasionally death. Although the characteristic features of lambda light chain paraprotein and mixed axonal/demyelinating neuropathy are highly suggestive of a POEMS syndrome diagnosis, misdiagnosis and diagnostic delay are very common. Over half of patients are initially thought to have CIDP, and thus receive ineffective courses of therapy with IVIG, plasma exchange and steroids with little or no improvement. In fact, such therapies are not without risk, in particular when provided to patients with cardiac and clotting abnormalities which can increase the risk of venous and arterial thromboembolism.[104] With such diagnostic uncertainties, median time to POEMS diagnosis is 15 months, by which time 35% patients are bed or wheelchair bound. Once such severe disability has developed, patients are less likely to functionally recover as well as someone identified sooner with less impairment.

Due to the rarity of POEMS syndrome, deep phenotyping of international cohorts to compare and contrast features is essential to our knowledge and management of the condition. The clinical characteristics and investigational findings from the UK cohort is compared to that of the Japan (n=102), USA (n=99) and China (n=99) below in table 2.4. Since the development of the UK POEMS registry, we have been able to uncover a range of important clinical aspects of POEMS syndrome, which would not have been possible without deep phenotyping . Examples of this have been characterising pachymeningitis

as an important clinical diagnostic clue,[1] detailing the endocrine features of POEMS syndrome,[95] identifying risk factors for disease progression and death,[3] optimising ASCT treatment and developing guidelines for thromboprophylaxis. [4,94]

Table 2.4: Comparison of clinical features of UK POEMS syndrome cases compared to Japanese, American and Chinese cohorts.

POEMS features	Number	%	Nakanashi % (n=102)	Dispenzieri % (n=99)	Li % (n=99)
Polyneuropathy					
Peripheral neuropathy	96	96	100	100	99
Raised CSF protein	65/65	100‡	97	100	96
Monoclonal plasma cell disorder	100	100	NA	100	100
Serum protein electrophoresis	55	55			
Serum immunofixation	78	78	75	85	92
IgM- λ	0	0‡	0	1	0
IgG- λ	34	44‡	54	48	22
IgA- λ	42	53‡	41	52	71
Biclonal	2	3‡			
IgG -κ	1	2‡	1	0	2
IgA- κ	0	0	4	0	1
Serum free light chain abnormal	65/83	78‡			
Kappa: lambda ratio					
Normal κ:λ	65	78‡			
>κ:λ	14	17‡			
<κ:λ	4	5‡			
Urine Bence Jones Protein	15	15			
Bone marrow abnormality	55/86	64‡			
0-4% PC	25	29‡			
5-9% PC	15	17‡			
10-14% PC	16	18‡			
Plasmacytoma biopsy abnormal	38/42	90‡			
Castleman disease	14	14	63	73	58

Bone lesions		81	81	55	97	27
	Solitary lesion	31	38†			
	2-3 lesions	20	25†			
	>3 lesions	30	37†			
	Sclerotic	45	55†	55	46	19
	Lytic	12	15†	14	2	8
	Mixed	24	30†	30	49	0
Vascular endothelial growth factor elevation		84/89	94‡			
Endocrine abnormality		68	68			
Organomegaly		63	63	82	50	86
	Splenomegaly	31	31	39	22	71
	Hepatomegaly	23	23	82	24	47
	Lymphadenopathy	42	42	65	26	75
Skin abnormalities		69	69	NA	68	90
	Hyperpigmented	25	25			
	Acrocyanosis	46	46			
	GH	23	23			
	Hypertrichosis	20	20			
	Skin thickening	14	14			
	Nail changes	12	22			
Extravascular volume overload		70	70	NA	29	88
	Peripheral Oedema	66	66	91	24	85
	Pleural effusion	16	16	40	3	43
	Ascites	6	6	62	7	55
Papilloedema		30	30	62	29	64
Thrombocytosis		34	34	NA	54	55
Polycythemia		18	18	19	18	9
Other						
	Weight loss	41	41			

Fatigue	22	22
Clubbing	10	10
Hyperhydrosis	12	12
VTE	34	34
Myocardial infarction	12	12
Stroke	9	9
Pachymeningitis	29/41	71†

Broadly, the clinical and investigational characteristics of POEMS syndrome are similar across the various international cohorts. This illustrates that although POEMS syndrome is complex with multiple disease features, presentation is in fact homogeneous at the level of individual patients. Features of disease such as the neuropathy, light chain clone and skin lesions are very typical and anomalies rare. Such findings may indicate that POEMS pathology is thus also consistent, homogeneous, and therefore reliable, albeit complex and currently not understood. Contrasting this, CIDP presents heterogeneously with several complex overlapping subtypes and is therefore considered to be an umbrella term for multiple pathogeneses resulting in a similar neurophysiological appearance of demyelinating neuropathy. Looking in detail at the frequencies across the cohorts, a lower frequency of organomegaly and higher rates of bone lesions separate POEMS syndrome in the UK cohort from China and Japan, but are more similar to the USA. Due to the retrospective nature of data collection in each cohort it may be that differences in the frequency of organ enlargement or bone involvement are secondary to the imaging used or perhaps how imaging was reported. Extravascular volume overload in the UK was present at a frequency much higher than the USA (70% compared to 29%) and more comparable to China. The difference in frequency between the USA and Chinese cohorts was initially assumed to be ethnicity related but is

evidently not the case. Fluid leak is thought to be driven by VEGF and/or other proinflammatory cytokines. It may therefore be of use to compare cytokine profiles across the two cohorts to establish whether underlying disease drivers differ. Another explanation may be of reporting bias due to different examination techniques or data collection proformas.

The key POEMS syndrome characteristics and their utility in diagnosis are elaborated upon in further detail below.

Neuropathy

The neuropathy in POEMS syndrome is typically of a subacute onset over months with numbness and dysesthesias followed by symmetrical, ascending, length-dependent weakness. Anecdotally, several patients in the cohort described the calf pain as a progressive muscular ache as if they had run too far. This was often misdiagnosed as claudicant peripheral vascular disease before the discovery of neuropathy. The clinical distribution of the weakness is typically distal at onset, with the frequent absence of proximal weakness, with other systemic features (such as unexpected volume overload in the young for instance) differentiating the condition from CIDP. Electrophysiology displaying a sensorimotor, symmetrical distal neuropathy with primary demyelination and secondary axonal loss is typical for POEMS syndrome, manifested by absent lower limb responses, and demyelination with or without mild early axonal loss in the upper limbs. [105] Because of the progressive and disabling nature of neuropathy, it is crucial to identify POEMS syndrome early in the disease course and initiate therapy to prevent

worsening disability. The combination of an inflammatory neuropathy and a lambda light chain monoclonal protein should serve as a 'red flag' for POEMS syndrome. Once identified, other features of POEMS syndrome should be sought, including the clinical features of oedema, skin changes and papilloedema, laboratory testing for VEGF and endocrinopathy, and imaging to identify bone lesions, organomegaly and nodal enlargement of Castleman Disease. As mentioned before, time to diagnosis in POEMS syndrome is a median of 15 months. Over time the neuropathy progresses, and patients go from distal foot numbness and weakness to being bedbound often with severe intrinsic hand muscle weakness and loss of function. Anecdotally, patients often experience slow and steady decline for several months, and then for reasons unknown, decline at a more rapid and worrying rate. Without knowing why this occurs, postulating the underlying neuropathology, this may occur once lower limb neurones go from a process of primary demyelination to reaching a critical stage of secondary axonal loss resulting in contributory muscle atrophy and worsening impairment. Knowing how or when this process occurs would be key to protecting neurological function.

From a clinical perspective, improving diagnosis in POEMS syndrome through recognition of the neuropathy at first presentation would improve time to diagnosis and prevent severe disability. In the majority of cases, POEMS neuropathy is misdiagnosed as CIDP and thus distinguishing the two is paramount to early identification of POEMS syndrome. In deep phenotyping the POEMS cohort we gained crucial experience in distinguishing the neuropathy of POEMS syndrome from that of CIDP. The key clinical and investigational findings that differentiate the two conditions are summarised below in table 2.5. Our clinical and economical cost analysis of a newly proposed diagnostic algorithm would also recommend early VEGF testing in all cases of acquired

demyelinating neuropathy to correctly identify POEMS cases, enabling prompt confirmation of the diagnosis and disease specific therapy. VEGF testing in this manner should be performed prospectively to support the data from our retrospective analysis.

Table 2.5: Key features differentiating POEMS syndrome from CIDP. Taken from "Keddie et al. POEMS neuropathy: optimising diagnosis and management".[46]

Assessment		POEMS syndrome	CIDP
History		Length dependent sensory and motor neuropathy	Patchy predominantly motor neuropathy with both proximal and distal features at onset
		Presence of pain typical (65%) [44]	Pain atypical
		Other multi-systemic symptoms present: Gynaecomastia Erectile dysfunction Weight loss Skin changes Oedema History of DVT	Multi systemic features less likely and unrelated
Examination	**General**	Finding of lymph nodes, skin lesions, organomegaly, papilloedema, peripheral oedema	Neuropathy likely in isolation
	Neuropathy	Dramatic distal weakness, predominantly UL flexors> ext. LL DF>>PF. Early distal wasting.	Evidence of proximal and distal weakness.
Investigation	**Blood/urine for monoclonal protein** [52,106]	Monoclonal IgA or IgG typical. IgM rare. Almost always lambda light chain	MGUS in 10-20%. Distribution of classes and light chains balanced. Consider MAG and GM1.
	Thrombocytosis [52]	Seen in 54% patients	Seen in 1.5%
	VEGF	Likely >2x ULN (771pg/ml)	Almost never raised. Unlikely >2xULN
	Neurophysiology [40,43,44]	Axonal and demyelinating. Preferential lower limbs, intermediate > distal nerve CV slowing. Conduction block (6%) and temporal dispersion unlikely.	Less axonal loss. Patchy proximal and distal demyelination. Conduction block (23%) and temporal dispersion more common.
	Nerve biopsy[18,44]	More axonal degeneration, diffuse myelinated fibre loss, increased epineurial blood vessels. Uncompacted myelin lamellae.	Endoneurial inflammation, multifocal myelinated nerve fiber loss.
	Imaging- xray/ PET/ CT	Sclerotic/ lytic bone lesions	Not routinely required.
		Pachymeningitis in approximately 70% of cases. Enhancing and enlarged nerve roots.	Pachymeningitis not seen- with neuropathy consider POEMS. Enhancing and enlarged nerve roots.
Treatment	**Steroids**	Mild response	Responsive*
	Immune therapy	Not responsive to IVIG/PLEX	Responsive to IVIG/PLEX*

CNS features of POEMS syndrome

Papilloedema, headaches and stroke are not uncommon central nervous system manifestations in POEMS syndrome. Our systemic analysis of 41 MRI brain scans revealed cranial pachymeningitis as a very common association in POEMS syndrome at 71%. This association has been previously documented in nine Italian patients, [107] but not identified in other cohorts or at this frequency.

The finding of cranial pachymeningeal thickening is not associated with CIDP, and therefore becomes a useful diagnostic clue when discovered. Due to its clinical utility and the high frequency seen in POEMS syndrome, we propose that (non-infective, non-malignant) cranial pachymeningeal thickening and enhancement should be included as a minor supportive POEMS diagnostic criterion. Other criteria for POEMS have been described iteratively from major cohorts and this finding is commoner in these patients than several other features.

Considering the underlying pathology of such a finding, although VEGF does not correlate with pachymeningeal thickening, Briani et al studied the histopathology of meningeal biopsy specimens taken from two patient with POEMS, two chronic inflammatory pachymeningitides, two from meningiomas and three healthy cases. They found hyperplasia of the meningoepithelial cells, neovascularisation and obstructed vessels without inflammation. VEGF and VEGF receptor were strongly co-expressed on endothelium, arterioles and meningoepithelial cells. This finding suggests meningeal thickening is a product of elevated cytokine levels, most possibly VEGF. The finding of

small vessel disease, being similar between CIPD and POEMS syndrome may be more related to other, non-disease specific risk factors such as age, smoking and immobility, for example. Secondary effects on renal and cardiac disease in POEMS syndrome may also contribute to hypertension and again increase the burden of small vessel disease.

Regarding the peripheral nerves, enlarged nerve roots were seen in 44% of cases. The initiating neuropathy in POEMS syndrome is distal, and thus proximal pathology is in some ways not expected, however is seen here at a high frequency and is a recognised finding in POEMS syndrome. Proximal nerve hypertrophy may be similar to that of pachymeningitis, in that cytokines (in particular VEGF) drive nerve endoneurial hyperplasia, vessel leak and oedema. This has been demonstrated in histopathological and nerve ultrasound studies of distal peripheral nerves in POEMS syndrome, [17,40,80] however the proximal nerves have not been examined. Although not explicitly mentioned, the proximal and distal pathology demonstrated in POEMS neuropathy is presumably either in fact demonstrated along the entirety of the nerve, or in areas of richer blood supply where cytokines are of higher concentration, or where poorer protective mechanisms of the blood nerve barrier exist. Certainly on nerve ultrasound, nerve oedema has been demonstrated at the wrist, forearm and elbow portion of the median nerve suggesting the pathology is uniform.[80] Understanding neuropathogenesis of POEMS syndrome also requires consideration of the blood nerve barrier biology and how it is disturbed in disease.

Monoclonal gammopathy of POEMS

Identification of a monoclonal plasma cell disorder in POEMS is crucial to the diagnosis. This study demonstrates the relatively poor sensitivity of serum protein electrophoresis at 55%. It is common practice for laboratories to perform immunofixation only if a paraprotein is present on SPEP, despite this and other studies indicating the superior sensitivity of immunofixation in detecting low level monoclonal bands missed by conventional electrophoresis techniques. [108] The disparity in SPEP and immunofixation techniques across laboratories, combined with differences in levels of sensitivity results in such tests often being difficult to interpret and rely upon. Not only that, detection of a monoclonal gammopathy does not always result in a POEMS diagnosis. The poor specificity of both tests often result in misclassification of the paraprotein as a coincidental MGUS, a paraprotein associated with CIDP or a non-POEMS related paraproteinaemic neuropathy. Serum free light chain analysis is often unrevealing with both light chains raised and a resultant normal ratio. Wang et al have demonstrated the largest proportion of bone marrow cells in POEMS syndrome are polyclonal despite the coexistence of clonal disease, [109] which would result in this more balanced light chain pattern, in comparison to more aggressive plasma cell malignancies such as in myeloma whereby the light chains of that monoclonal plasma cell are often significantly raised. Fifteen cases in fact demonstrated a raised kappa, rather than lambda light chain with normal lambda, however in such cases, the kappa light chain level was often minimally elevated over the upper limit of normal, and kappa to lambda ratio remained small at 1.8 suggesting again polyclonal increase of both light chains rather than a significant predominant kappa chain. This study also demonstrated that serological investigations

alone do not suffice, whereby urinary Bence Jones protein or light chains were detectable in 5% with normal SPE and IF on blood testing.

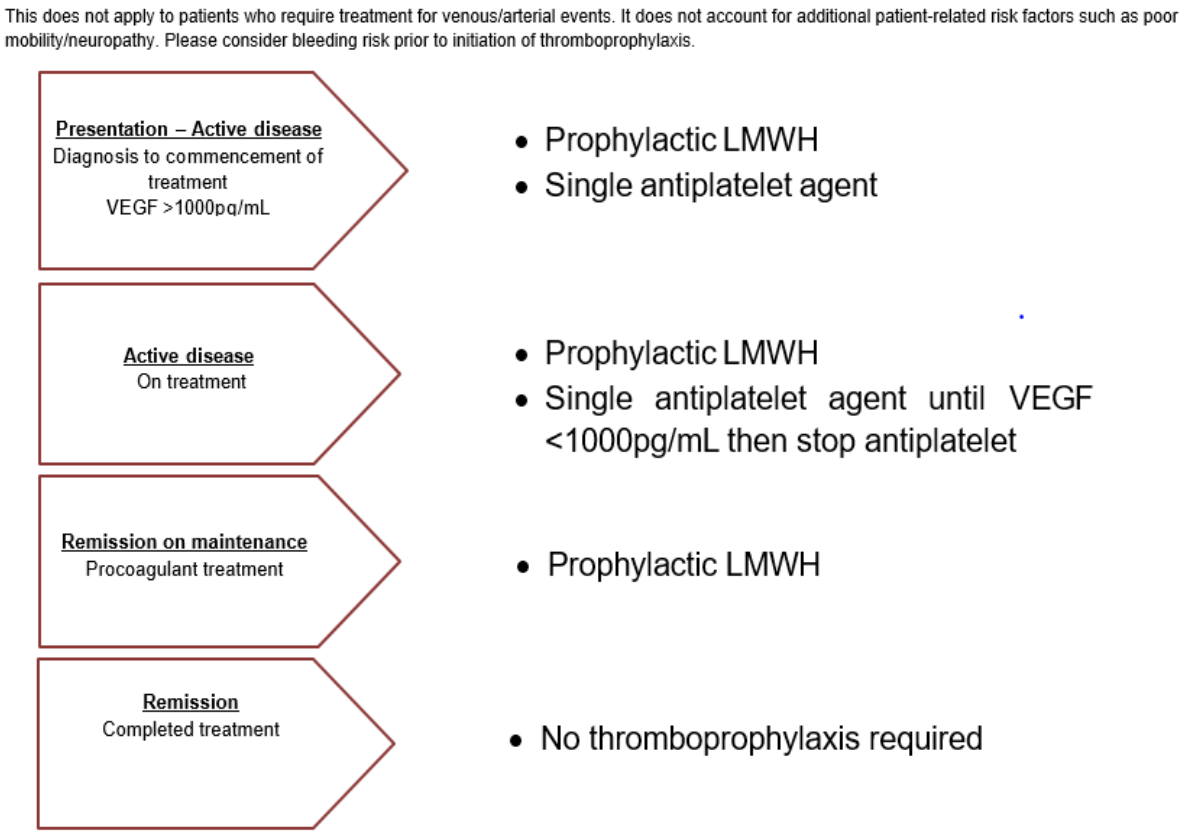
VEGF as a diagnostic marker of POEMS syndrome

The poor sensitivity and specificity of SPE and IF in detecting a monoclonal gammopathy and diagnosing POEMS syndrome has been clearly demonstrated by this retrospective cohort analysis. This study in combination with previous published data by our team has demonstrated VEGF as a highly sensitive and specific biomarker in diagnosing POEMS syndrome, [12] which is relatively simple to interpret and rule out false positives. Our costing analysis has demonstrated that the current standard of practice, which relies upon determination of the classic lambda light chain restricted paraprotein for a diagnosis of POEMS syndrome resulted in misdiagnosis in 55% of cases, and between £808,550 and £1,111,756 on provision of ineffective CIDP treatments. Also, time lost at employment or education, travel costs for treatment, and the emotional and social impacts of diagnostic uncertainty are equally significant indirect costs. This analysis has demonstrated an improved rate of diagnosis and cost saving of £107,398 through incorporating mandatory IF testing with all SPE tests, and with VEGF testing upon determination of an acquired demyelinating neuropathy, particularly those considered to have CIDP whereby treatment is to be given. A significantly raised VEGF at this stage should prompt thorough exploration for an underlying monoclonal plasma cell disorder if not already discovered upon initial serological testing.

Thrombosis in POEMS syndrome

This cohort analysis has demonstrated high rates of both arterial and venous thromboses occurring in POEMS syndrome. Arterial events were most likely to occur pre-treatment, and always in the context of raised VEGF >1000 pg/ml. Venous events occurred both pre-treatment and during therapy with drugs which are known to increase the risk of venous thromboembolism. Importantly, 20/27 patients were not taking any form of thromboprophylaxis when an event occurred thus demonstrating the requirement of a change to management. The data from this study prompted the POEMS team to collaborate with clotting specialists at UCLH to propose new thromboprophylaxis guidelines for patients with POEMS syndrome from diagnosis to maintenance therapy, shown below.

Figure 2.8 Newly proposed thromboprophylaxis guidelines for POEMS syndrome patients



Treatment of POEMS syndrome

Survival in treated POEMS syndrome patients is favourable. ASCT demonstrates the most improved PFS and OS when compared with other treatment modalities, but this may be in part due to selection bias of treating more systemically well patients. Several studies have recently demonstrated significant haematological and VEGF response following treatment with lenalidomide, [37,58] including in patients originally deemed unfit for ASCT who then underwent ASCT at later date, [31] and in relapsed disease. [110] Twenty-six of 100 patients were deemed unfit for ASCT first line so received lenalidomide treatment in this cohort, of which eight went on to receive ASCT successfully (table 2.3). In addition, prior to 2018, routine practice was to perform up front ASCT in systemically 'well' patients (meaning no pre-transplantation chemotherapy). The rationale was because POEMS manifests typically with subtle bone marrow changes, so it was therefore deemed unnecessary to strive for a deep haematological response following first line therapy, and ASCT would suffice. However, after reviewing our data on risk factors associated with POEMS outcome, and studies conducted demonstrating significantly improved outcomes on pre-treated cases, [31,36] we now provide 6 months of lenalidomide therapy to all cases planned for ASCT. This management strategy is effective in that it is easy to start, is well tolerated, simply monitored and often results in significant improvement in patients' haematological and VEGF indices and clinical state (particularly fluid overload), as a pre-optimisation step for ASCT. It is too soon to definitively report whether such alterations in management have resulted in more favourable outcomes. More analysis is required to define lenalidomide's effectiveness in different treatment regimens.

Risk factors, progression free and overall survival in POEMS syndrome

Low albumin at presentation, non-treatment with ASCT, non-haematological and non-sVEGF response following therapy are all significant factors predicting the risk of progression or death in POEMS syndrome. Low albumin is possibly caused by a variety of factors including poor nutritional state, renal insufficiency and the impact of VEGF on blood vessel permeability. Failure to achieve haematological or VEGF response suggests persistence of pathological drivers in POEMS syndrome and therefore treatment failure, both of which demonstrated high hazard ratios towards progression or death. As mentioned previously, the true benefit of ASCT in preventing progression or death is difficult to quantify as younger patients with fewer comorbidities are selected for ASCT and thus may skew results towards favourable outcomes. Utilising the multivariate model allows for easily applicable risk score quantification. This may be useful in the monitoring of POEMS patients through identification of high-risk cases, prompting closer follow up and more aggressive treatment strategies. For low-risk patients, a 'watch and wait' approach may be more appropriate. For reasons unknown, there are a proportion of patients who are poor responders to ASCT with persistent monoclonal proliferation and VEGF response. Prior to the above discovery, it was unknown whether the persistence of a paraprotein post-treatment was of relevance. It was difficult to rationalise further therapy, particularly when patients with clear biochemical and haematological activity were clinically well. Now we know these patients are highly likely to relapse, further therapy to suppress persistent clones and VEGF is therefore advisable in some cases.

To summarise, this detailed cohort analysis has not only become a useful repository of data and samples for this and subsequent research projects, but has also developed our understanding of the clinical characteristics and investigation findings of POEMS patients. It has improved our ability to diagnose patients sooner, identify risk factors for poor outcomes and optimise treatment strategies.

3. Biomarkers in neuropathy

3.1 Introduction

The National Institutes of Health Biomarkers Definitions Working group defines biomarkers as "objective measurements that act as an indicator of normal biological or pathological processes, or pharmacodynamic responses to therapeutic intervention".[111] Serum and CSF biomarkers are of considerable research interest, particularly in the field of central nervous system neurodegeneration, which has enabled more accurate measurement of disease severity and prognostication.[112] Specific biomarkers in peripheral nerve neurology have however been lacking, and existing clinical and neurophysiological measures are not without limitation. There is a compelling need for accurate and reliable biomarkers in peripheral nerve neurology to aid with diagnosis and the understanding of peripheral nerve pathogenesis, prognostic quantification and measuring response to treatment.

The following chapter critiques the current available methods of peripheral nerve disease measurement, with a focus on POEMS neuropathy, and argues for the need of more accurate and reliable markers. It then goes on to outline the desired characteristics of an effective biomarker in clinical practice, and describes modern technological advances to allow for such measurements to be made. The experimental data of this chapter detail the development of a novel peripheral nerve biomarker, peripherin, for the measurement of axonal neuronal pathology. The development of this assay from conception, to pre-analytical validation and testing clinical samples will be detailed and discussed.

3.1.1 Existing biomarkers of peripheral nerve disease

3.1.1.1 Clinical outcome measures

Clinical outcomes can be used to quantitate the severity of peripheral nerve disease. Several neuropathy severity scales exist, such as the Overall Disability Sum Score (ODSS), the Rotterdam Inflammatory Neuropathy Cause and Treatment Scale (INCAT), the Overall Neuropathy Limitation Scale (ONLS) and the Rasch-built Overall Disability Scale (RODS). Outcome measures can quantify impairment, activity, limitations and participant restrictions as well as quality of life.[113] Classical test theory derived ordinal scales lack linearity, sensitivity to change, and reliability.[114] Rasch-built models address issues with linearity of disease measurement but remain disease specific and therefore those that exist so far are not entirely applicable to POEMS syndrome. For example, the Rasch-built CIDP i-RODS score is validated for patients with CIDP, which typically affects proximal muscles early in disease, and thus a proportion of questions in the CIDP-iRODS are set to measure outcomes related to proximal weakness. POEMS neuropathy is very length dependent in nature, and thus measures of proximal strength or function may be less relevant. As POEMS is a multi-system disease, the influence of other organ systems may modulate disability and interfere with questions designed around a primary nerve disease. Outcome measures which do not rely on patient involvement typically require physician input for grading either functional ability or examination features. This is often unreliable, particularly with inexperienced operators. [115] A more objective, reliable and robust marker is required to assist with measuring peripheral nerve disease more accurately.

3.1.1.2 Neurophysiology

Neurophysiology of peripheral nerve conduction studies and electromyography is an extension of the neurological examination. The determination of conduction slowing, a surrogate marker of demyelination, versus reductions in amplitude, suggestive of axonal loss, is fundamental to understanding pathological processes and promptly focuses the differential diagnosis. Neurophysiological studies require specialist training and experience to perform, are time consuming and often painful for patients. For that reason, performing several neurophysiological studies in quick succession in an attempt to measure disease over time is not feasible. Patient dependent, technical and environmental factors also affect results.[116] Furthermore, the interpretation of neurophysiological data is complex, requires time and specialist training and is only semi-quantitative. Interpretation can be at risk of inaccuracy and inter-observer discordance. For example, with loss of myelin which occurs in a number of acquired neuropathies such as Guillain Barré Syndrome (GBS), CIDP, paraproteinemic neuropathies and inherited disorders such as Charcot-Marie-Tooth disease, the nerve conduction velocities are slowed.[117] However, such conduction slowing is also seen in axonal pathologies with loss of the fastest conducting large fibre axons, and therefore studies with slowed conduction velocity are often misreported as displaying 'demyelinating pathology' when in fact the primary pathology is axonal. In cases where demyelination is severe enough to the point of failed saltatory conduction, conduction block occurs. This is measured neurophysiologically by a reduction in the compound motor action potential (CMAP) area with stimulation proximal to the site of block than that stimulated distally. However in conditions in which demyelination occurs proximally, such as GBS, it may not be possible to stimulate proximal to the site of block,

and demonstrate conduction blockade. Thus, the combination of technical, operator and analytical challenges limit the utility of neurophysiology despite it currently being the 'gold standard' of nerve pathology measurement.

3.1.1.3 Peripheral nerve imaging

Imaging though high-resolution ultrasound (HRUS) and MR-Neurography (MRN) are increasingly used for the evaluation of the peripheral nervous system. Although imaging may detect enlargement and/or enhancement of nerves in POEMS syndrome and that of other inflammatory neuropathies, improved techniques and interpretation are required to differentiate the dysimmune neuropathies solely by imaging modalities, and scoring tools are currently being developed using pattern analysis to diagnose precise neuropathologies. [118] To date, imaging has been more successful in determining response to treatment with pre- and post-treatment images displaying changes in the structural appearance of nerves. MRI is relatively high-cost and time consuming compared to HRUS, which also has an advantage of dynamic imaging examination, however HRUS requires considerable training to perform and interpret, with significant issues with interrater reliability depending on examiner experience.

3.1.1.4 Fluid biomarkers

Fluid biomarkers involve the measurement of a change in expression or state of a protein that correlates with the risk or progression of a disease, or response to treatment.[119] Rapid scientific and technical advances have enabled reliable and

affordable measurement of novel fluid biomarkers which assist assessment and management of patients with neurological disorders beyond current practice standards.[120,121] Such biomarkers have been useful in capturing the different aspects of disease heterogeneity and pathogenesis, helping characterise patients, inform targeted treatment, and measuring response to interventions. Some examples of such are below:

- CSF T-tau, P-tau and Aβ42 in the diagnosis of Alzheimer's disease, correlating with degree of cognitive impairment.[122]
- CSF and serum neurofilament (NfL) levels in the diagnosis and disease severity of a range of central nervous system disorders such as Alzheimer's, multiple sclerosis, Parkinson's, Huntington's and traumatic brain injury.[123]
- Glial fibrillary acidic protein measurement for early diagnosis of intracerebral haemorrhage.[124]
- Anti-NMDA receptor antibodies in the diagnosis of NMDA receptor encephalitis and titres correlating with poor outcome.[125]

Fluid biomarkers have a number of inherent advantages which are discussed in detail in section 3.1.2. There are two fluid biomarkers which appear of relevance in measuring disease in POEMS syndrome and related neuropathies; neurofilament light and vascular endothelial growth factor, and these will be discussed below.

The technological development of highly sensitive immunoassays have accelerated the field of biomarker discovery and development. In particular, in some instances this has allowed for the availability of biomarker testing in blood-based assays which is of considerable benefit, notably in neuromuscular disease. The experimental techniques

utilised to achieve such unprecedented levels of sensitivity, in particular that of Single Molecule Array (SiMoA) are described in section 3.1.3.

3.1.1.4.1 Neurofilament Light

Neurofilament light (NfL) is a neuronal cytoskeletal protein expressed in large calibre myelinated axons. CSF and blood levels of NfL increase proportionately relative to the degree of axonal damage in a broad range of neurological pathologies including inflammatory, neurodegenerative and traumatic. NfL is measurable in a range of acquired and inherited peripheral neuropathies, including GBS, CIDP Charcot-Marie-Tooth, and hereditary amyloidosis, and these are discussed below.[126–128]

Guillain-Barré Syndrome

Petzold et al demonstrated significantly raised levels of CSF neurofilament heavy using ELISA testing in GBS cases, correlating with disease severity and poor functional outcome.[129,130] Altmann *et al,* in collaboration with our laboratory have recently published a study which measured serum neurofilament light (NfL) using SiMOA in 27 GBS cases compared to controls. [131] They found baseline NfL levels in GBS cases to be 85.5 pg/ml compared to 9.1 pg/ml in controls. In addition, a baseline two-fold increase of NfL was associated with a Hughes functional outcome scale (HFS) (ranging from 0, no symptoms, to 5, requiring ventilation) increase of 0.6 at nadir, and reduced the likelihood of unimpaired walking ability by a factor of three. In addition, higher NfL levels on admission predicted the need for intensive care support with an odds ratio of 2.4. Querol et al have furthermore demonstrated serum NfL levels to be increased in

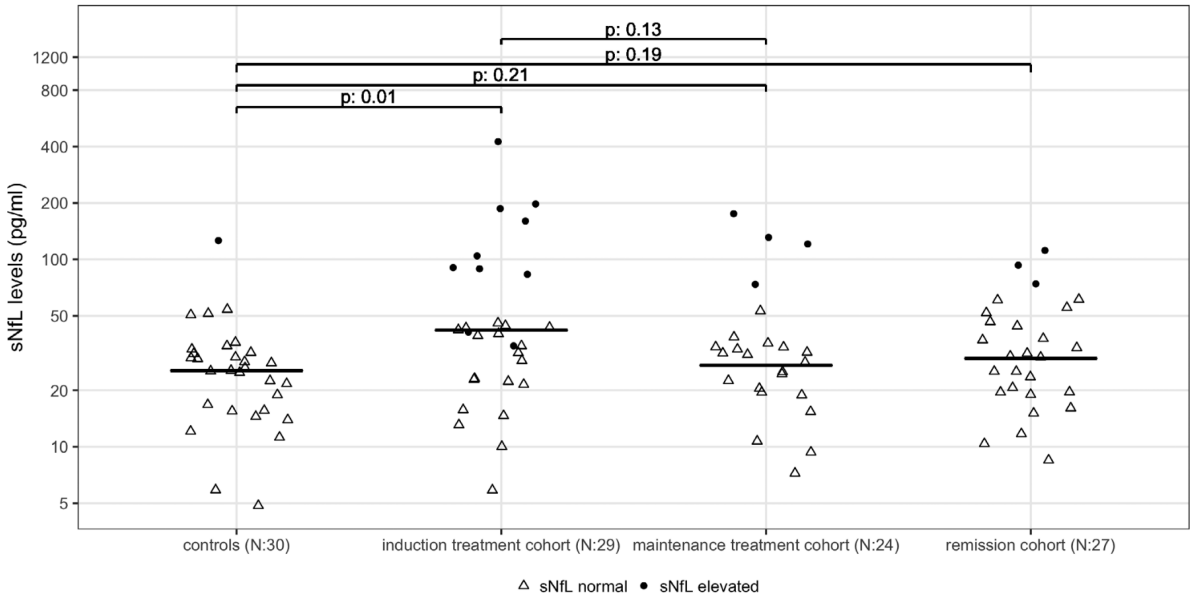
GBS cases using SiMOA, in particular with axonal subtypes, with levels correlating with GBS disability score and the i-RODS GBS, and predicting outcomes such as the ability to run at 6 months. They also found that NfL levels of >319 pg/ml were predictive of the inability to walk independently at one year.[132]

Chronic Inflammatory Demyelinating Polyradiculoneuropathy

NfL has also been studied in CIDP which is often clinically less severe with less axonal loss. One study stratified patients into three groups; (a) patients starting induction treatment (IT cohort, N=29) measured at baseline and 6m post treatment, (b) patients on maintenance therapy starting IVIG withdrawal (MT cohort, N=24) measured at baseline and 6m post withdrawal, and (c) patients in long term remission without treatment (N=27) using SiMOA (see figure 3.1). At baseline, serum NfL was higher in patients starting IT than healthy controls. Patients with active disease (non-responders and patients who relapsed after IVIG withdrawal) had higher sNfL levels compared to patients with stable disease (responders and those successfully withdrawn from IVIG).
[133]

Figure 3.1: Serum NfL levels in patients with CIDP.

Taken from van Lieverloo et al [133]



Charcot-Marie-Tooth

Rossor et al demonstrated plasma NFL to be significantly higher in patients with Charcot Marie Tooth (CMT) compared to healthy controls, which correlated with disease severity measured using the Rasch modified CMT examination.[127] The authors highlighted a limitation that in most cases, the NfL concentration difference between healthy controls and disease cases is very subtle. NfL also increases with age and therefore overlap often exists between 'normal' healthy cases and disease (see figure 3.1 as an example). Statistically significant differences can be achieved when grouping large numbers of cases together, however interpreting individual values from patients are of limited clinical utility.

Hereditary transthyretin amyloidosis (ATTR)

Kapoor et al, again from our laboratory, measured NfL in 73 patients with ATTR and found that levels were significantly raised in patients with neuropathy compared to healthy controls. Furthermore, NfL also correlated with disease severity as defined by the neuropathy impairment score. This finding is of clinical relevance, as several current treatments are only licenced for ATTR patients with evidence of peripheral neuropathy, and therefore would be a useful criterion for instigating therapy.[134]

POEMS syndrome

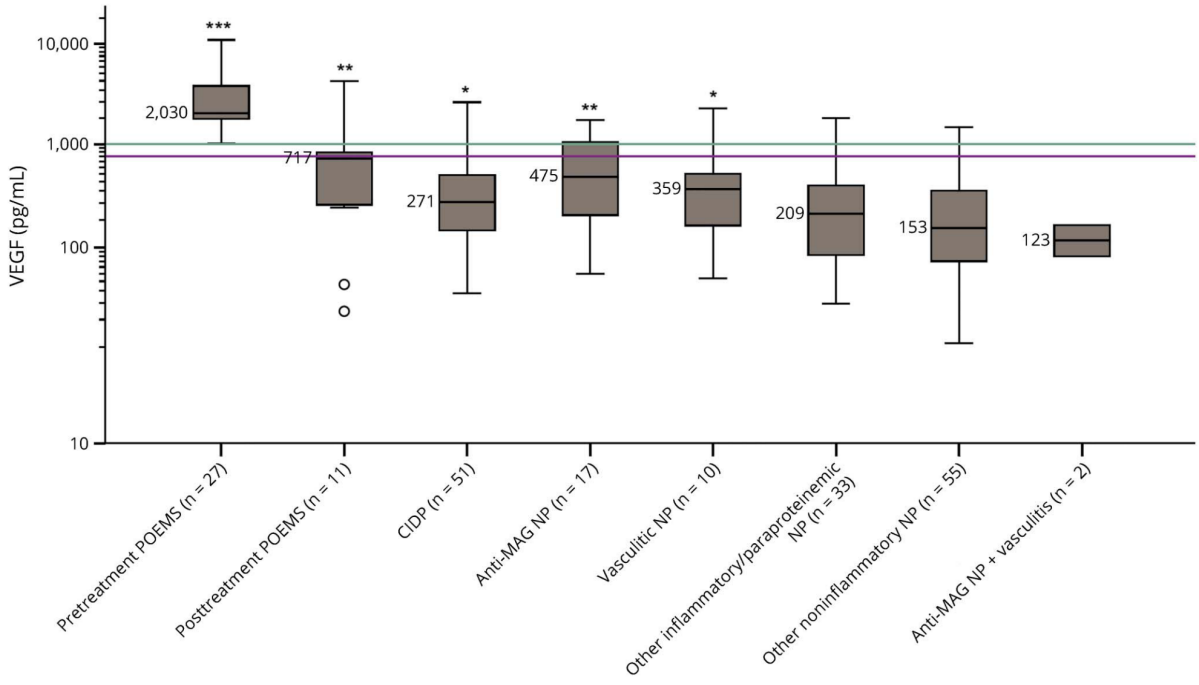
To date, there have been no studies investigating neurofilament levels in POEMS syndrome. Neurophysiology in POEMS syndrome is thought to represent primary demyelination with secondary axonal loss. The axonal loss is thought to result in irreversible nerve damage, and thus correlating NfL levels with functional markers of neuropathy severity and outcome would be of potential clinical utility. Also, POEMS syndrome has highly effective therapies which are thought to 'switch off' underlying disease. It would therefore be of use to study NfL levels in pre- and post-treated patients, serially over time to determine whether treatment abates ongoing axonal loss, justifying the requirement of early identification and treatment.

3.1.1.5 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) is a potent proinflammatory cytokine which is significantly raised in patients with POEMS syndrome compared to other

haematological malignancies and inflammatory neuropathies. We tested a cohort of 195 consecutive samples with neuropathy or non-POEMS haematological disease using a commercial ELISA assay kit. This study demonstrated that serum VEGF (sVEGF) is 100% sensitive, and 91% specific to the diagnosis of POEMS syndrome in patients with a neuropathy, and 92% in those with paraproteinaemia (figure 3.2).[2] Raised VEGF is now a key diagnostic criterion of POEMS syndrome, and highly useful clinically. Multiple regression analysis identified iron deficiency anaemia, chronic obstructive lung disease (COPD) and obstructive sleep apnoea (OSA) as alternative causes of elevated sVEGF (>1000). The response to treatment in VEGF is also an independent predictor of overall and progression free survival (figure 2.7).[13,33,38,135] VEGF however does not correlate with the degree of disease activity or severity of neurological disability, where patients with markedly elevated VEGF levels can have minimal systemic features and mild neuropathy, with excellent outcomes, and vice versa (as described in chapter 2). For this reason, although VEGF is diagnostically useful and complete VEGF suppression following therapy is a strong predictor of progression free and overall survival, no predictions can be made with regards to patients' level of function or likely neurological recovery using VEGF. This finding does not support the hypothesis of VEGF causing BNB breakdown and neuropathy in a dose-dependent fashion.

Figure 3.2: Serum VEGF concentrations in different neuropathy groups



sVEGF is significantly raised in POEMS patients compared to other forms of peripheral neuropathy and haematological malignancy. Taken from Phian M, Keddie S et al "Raised VEGF- high sensitivity and specificity in diagnosis of POEMS syndrome".[2]

3.1.2 Features of an optimal peripheral nerve biomarker assay

Knowledge of the structure, function and dynamics of a biomarker is essential to designing an instrument of measure. This is of particular relevance in the nervous system, which is a structurally and immunologically privileged site, and therefore a number of factors need to be taken into consideration. Features of an optimal peripheral nerve biomarker assay are listed below, and some of the key pre-analytical considerations, based upon knowledge of other neurological biomarkers, are discussed in more detail.

Pre-analytical features

- Samples are simple to collect through a minimally invasive technique
- In disease, the biomarker is distributed with sufficient abundance in the sample matrix for measurement
- Samples are relatively simple to measure without several pre-procedural steps
- Samples can be stored with minimal degradation over time

Analytical features

- The biomarker assay should display valid spike recovery, parallelism, precision and dilution linearity

Clinical validation

- The biomarker clearly discriminates healthy from disease
- The biomarker is able to accurately detect underlying axonal (or demyelinating) pathology
- Levels of the biomarker correlate with severity
- The assay is able to produce reproducible results

A number of the features are discussed in more detail below.

3.1.2.1 Sample collection

Until now, most neurological biomarker assays for neuronal damage are taken from the cerebrospinal fluid (CSF) to measure central nervous system (CNS) diseases. This is intuitively sensical since the brain is bathed in CSF and therefore CNS damage markers are released directly into the overlying fluid, whereas they would only enter the systemic circulation after resorption of CSF through arachnoid granulations or transit through a damaged blood brain barrier. A lumbar puncture procedure is however required for CSF collection. This requires specialist training to perform, is time consuming and costly. The procedure is sometimes painful for patients, and can have adverse effects, ranging from the very common post lumbar puncture headache to rarer and more dangerous such as meningitis, epidural haematomas and subarachnoid haemorrhage.[136] This therefore practically and financially restricts the number of samples that can be collected, in particular the number of longitudinal samples that can be collected from individual patients. In comparison, testing blood samples is more advantageous as venepuncture can be performed more frequently, with relatively little procedural training and minimal complications.

3.1.2.2 Biomarker distribution

Peripheral nerve fascicles contain blood capillaries that make up the vasa nervorum in which peripheral nerve biomarkers are released, which makes blood, rather than CSF the optimal sample matrix of measurement. PNS disorders which cause damage to proximal nerve roots located in the subarachnoid region can release biomarkers into the CSF, such as in GBS.[137] Querol *et al* measured NfL in both the serum and CSF of GBS

cases and found the difference in levels of NfL between GBS cases and healthy controls was greater in serum samples compared to CSF, suggestive that in fact the greater number of affected axons are in the periphery.[132] A large proportion of PNS disorders are length dependent, or distal predominant, and thus rarely involve the proximal nerve root. CSF may therefore not be a suitable sample matrix except where proximal damage can be detected or is more important. Therefore, measuring PNS biomarkers using blood samples appears more likely to yield meaningful results. Serum and plasma however contain globular proteins which can interfere with the measurement of low abundance biomarkers through matrix effects, inhibiting the interaction of the biomarker protein with its target. This is compared to CSF which is almost protein free with only 0.3% plasma proteins.[138] This limitation can be accounted for during assay development through optimisation of calibrator and sample diluents to minimise such effects.

3.1.2.3 Biomarker abundance

The main limitation of measuring serological peripheral nerve biomarkers is the abundance of protein in the sample matrix. The tissue volume of the peripheral nervous system is far less than that of the brain, and thus damage to the PNS is likely to result in significantly lower amounts of biomarker release. Secondly the volume of distribution of biomarkers in the blood circulation volume (5 litres) is far higher than that of the CSF (200 ml), resulting in expected concentrations of blood-based biomarkers to be in the picogram/ml range and therefore requiring highly sensitive methods of detection.

Before optimisation of a biomarker assay, it is important to determine the required sensitivity of the assay, which will influence the methodology and development. There are no prior data available regarding the expected concentration of peripheral nerve biomarkers in the blood or CSF. NfL has been the most promising and reliable biomarker of study for neurological axonal damage to date, which provides an indication of biomarker abundance and thus required assay sensitivity. [123] In a study of NfL in a range of PNS disorders (GBS, CIDP, multifocal motor neuropathy (MMN), vasculitic neuropathy and anti myelin-associated glycoprotein (MAG) neuropathy), serum NfL levels were recorded at 31.52 pg/ml, compared to 1407 pg/ml in the CSF, but healthy controls were not included for comparison. Different final diagnoses did not influence serum/CSF NfL values, suggesting that distal diseases still result in protein leak into the CSF. [139] In the Querol *et al* study, serum levels of NfL in GBS were 55.5 pg/ml compared to 9.8 pg/ml in healthy controls. In addition, in normal populations, NfL levels increase as people age, particularly in those over 60 years.[140] These studies illustrate the necessity of highly sensitive assays with a detection level accurately and reliably in the pg/ml range to produce clear distinctions between groups with very low-level biomarker levels to distinguish diseases and identify normals. For this reason, the measurement of serum NfL, and thus likely levels of other peripheral nerve biomarkers have not been achievable previously, until more recently with the introduction of highly sensitive immunoassay techniques, which are discussed in the next section.

3.1.3 Selecting an appropriate immunoassay technique

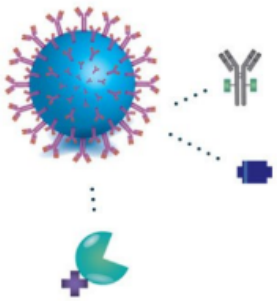
NfL has been quantified in the blood using enzyme-linked immunosorbent assay (ELISA) and more sensitive electrochemiluminescence (ECL) assay technology in

several diseases. [141,142] Neither technique has been able to detect small disease related changes which are fundamental to biomarker's functional ability and clinical application. Only the introduction of novel immunoassays such as O-Link and single-molecule array (SiMOA) assays have enabled such reliable detection in the blood.[143] Kuhle and Zetterberg *et al* demonstrated this in a study conducted in 2016 which compared ELISA, ECL and SiMOA's ability to detect NfL in CSF and serum samples. The lower limit of sensitivity was 0.62pg/ml for SiMOA, 15.6pg/ml for ECL and 78.0pg/ml for ELISA. [144] This is important as the NfL level for healthy controls is close to the limit of detection in ECL and thus several samples will inevitably be under the measurable sensitivity of the assay. The commercial NfL kit now has an unprecedented lower limit of detection of 0.038pg/ml.[145] SiMOA immunoassays are also relatively simple to set up (once the paramagnetic beads and biotinylated detector is produced), can run large sample numbers in each run due to the automated function of the instrument (and can therefore be left overnight), and often display low coefficient of variance values between samples and controls due to the machines' automated sampling method.

SiMOA technology utilises several in-built mechanisms to amplify signal. The immunoassay begins with a paramagnetic bead coupled with the capture antibody selectively binding to the antigen/biomarker of choice. This antigen is then detected by a biotinylated secondary antibody to the antigen to form a captured analyte sandwich, and the immune-sandwich is then labelled with an enzyme conjugate (streptavadin β galactosidase, (SBG)). Following incubation steps, the immunobead is then re-suspended in a resorufin β-D-galactopyranoside (RGP) substrate. The beads are then transferred to the entry port of a SiMOA disc for imaging and analyte quantification. The

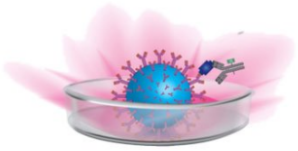
disc itself is the solid platform of the array, and contains 238,000 wells that have been designed to hold no more than one bead per well. A vacuum pulls the beads into each well. Oil is spread over the surface of the array, removing excess beads from the array surface and sealing loaded wells. The instrument camera then images the sealed wells, capturing the florescence emitted by the enzymatic product of the labelled immunocomplexes. The instrument determines the number of wells which have a florescent bead in an 'on-off' binary fashion. At higher concentrations there can be several immunocomplexes bound per well, producing greater signal per well and triggering the analogue counting phase. These two modes facilitate a larger dynamic range to be achieved with this platform compared to conventional immunoassays. [146] This process is illustrated below in figure 3.3.

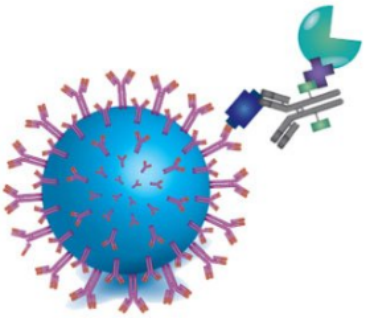
Figure 3.3: Five steps of Single Molecule Array for highly sensitive biomarker detection. Figure adapted from Quanterix homebrew assay development guide. [146]



Step 1: Paramagnetic bead coupled with capture antibody designed to bind to specific antigen epitope is added to serum sample

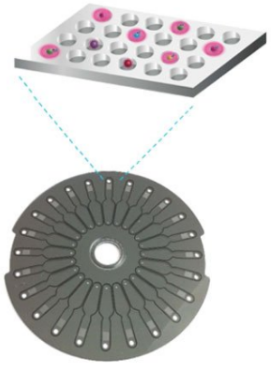
Step 2: Biotinylated detection antibodies bind to specific antigen epitope.





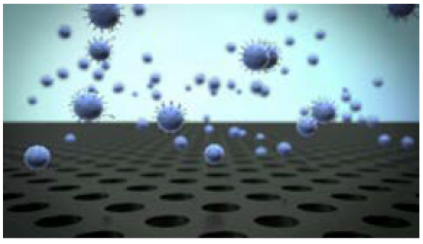
Step 3: Streptavidin β galactosidase (SBG) enzyme conjugate binds to biotinylated detector.

At low concentration, each bead will contain one bound protein, or none



Step 4: The sample is loaded into arrays in the SiMOA disc consisting of 238,000 microwells. Each well can hold one bead.

Load



Step 5: A vacuum pulls the beads into each well, oil is delivered and the instrument camera images the wells.

3.1.4 The potential applications of peripheral nerve biomarkers

3.1.4.1 Diagnosing peripheral nerve disease and differentiating from CNS pathology

The potential and actual clinical applications of peripheral nerve biomarkers are many. Firstly, and of similar clinical utility to Nfl, a peripheral nerve axonal biomarker should assist in the identification of peripheral neuropathy of various causes. The severity of peripheral nerve damage should also be reflected by the concentration of the biomarker in the blood, and thus give an indication of the underlying degree of active axonal loss. A limitation of the current 'gold standard' PNS biomarker, NfL, is that it is an axonal neurofilament which is ubiquitous to all central and peripheral axons. NfL levels for example correlate with age, particularly with a steep increase in over 60's, likely related to normal cerebral atrophy over time.[123] This means that measuring PNS disease with a marker of CNS and PNS pathology can result in inaccurate and potentially misleading results. Previous studies measuring serum NfL in PNS disease have noted high variability among patients, with levels ranging from normal levels to 100-fold higher than that of healthy controls.[132] This may be due to CNS derived neurofilament confounding results. The advantage of a peripheral nerve specific biomarker is that levels of the biomarker will not be influenced by CNS pathology. Although peripheral nerves will degenerate at a similar degree, a peripheral nerve specific biomarker will not only provide a more accurate reflection of PNS damage, but may also be used to quantitate the relative proportion of PNS damage in conditions in which pathology occurs to both the PNS and CNS, such as amyotrophic lateral sclerosis and ALS-frontotemporal dementia. Such potential applications have already sparked the interest

of research groups interested in MS, ALS, frontotemporal dementia and Huntington's disease.

3.1.4.2 Differentiating axonal from demyelinating pathology

In theory, one should be able to quantitatively measure the relative proportion of axonal and demyelinating involvement in a range of peripheral nerve disorders. As mentioned before, identification of primary axonal versus demyelinating pathology in peripheral neuropathies enables clinicians to focus the differential diagnosis. Such biomarkers could also enable greater understanding of underlying peripheral nerve pathogenesis, and may confirm or refute what is already understood from electrophysiological and histopathological findings. Examples of how this could be implemented are below.

- In cases in which neurophysiology is difficult to interpret, such as slowed conduction as a result of large fibre loss or proximal conduction block, biomarkers could be interpreted in conjunction to clarify the underlying pathology.
- GBS results in a highly variable clinical course and outcome, and in some cases leads to permanent ventilation, tetraparesis and death. [147–149] Large prospective and retrospective analyses from the International GBS Outcome Study (IGOS) have been undertaken to predict factors contributing towards clinical outcomes such as requirement for mechanical ventilation in the first week of admission and ability to walk at 6 months. [150–152] Predicting disease course through measurement of clinical indicators may also be supplemented with fluid

biomarkers. Patients with very high levels of axonal biomarker release early in disease onset are more likely to have severe disease and poor outcomes.[131,132]

- Subtypes of GBS respond differently to treatment and have different prognoses. Such subtypes are classified based on the underlying peripheral nerve pathology, which could be easier to identify using diagnostic biomarkers. For example, acute inflammatory demyelinating polyneuropathy (AIDP), the most common variant in the Western world, is defined by demyelination, mostly without axonal damage. In other forms, axonal degeneration predominates (acute motor axonal neuropathy, (AMAN) and acute motor and sensory axonal neuropathy, (AMSAN)).[147] Knowing which underlying pathology predominates may serve for more accurate neuroprognostication.
- Differentiating POEMS syndrome from CIDP is crucial towards providing disease specific therapy and improving outcomes.[5] Neurophysiologically, POEMS syndrome displays significantly more axonal loss than CIDP (particularly early onset), which may be well characterised through biomarker measurements as neurophysiology is often misinterpreted, and thus misdiagnosis common.[5]
- Diagnosis is paramount to conduct successful drug development trials, whereby it is critical to ensure subjects enrolled are those with an accurate clinical diagnosis and are therefore correct candidates for the treatment selected. As mentioned previously in inherited amyloidosis, for example, several current treatments are only licenced for patients with evidence of peripheral neuropathy.[134]

3.1.4.3 Monitoring disease activity, progression and prognosis

Measuring activity in peripheral nerve disease is fundamental to monitoring, prognosticating and making important treatment decisions. This requires detailed interval neurological examination or clinical outcome scores to detect meaningful changes. However, appropriating scoring systems not validated for the disease in question may not accurately capture severity or be sensitive enough to detect minimal changes. For example, lack of neurological improvement following POEMS treatment is ambiguous and could either be due to lack of response to treatment, or (more often the case) a delayed response before significant, measurable improvement is demonstrated. Such misinterpretation occurs due to inherent limitations of neurophysiology and clinical examination as monitoring tools of peripheral nerve disease. This presumed lack of therapeutic response may prompt physicians to deliver further unnecessary and potentially harmful lines of therapy. A sensitive biomarker of peripheral nerve damage would potentially assist with such monitoring, whereby levels of ongoing damage rather than levels of activity/function are measured. One would assume that following treatment, biomarkers of damage would drop. Thus, an objectively measurable change in a direct biomarker would potentially be recorded earlier than our current monitoring tools would allow.

Axonal loss in neuropathy often signifies considerable irreversible damage, and thus poor prognosis. This has been clearly identified in primarily demyelinating pathologies such as CIDP.[153] Neuropathy in POEMS syndrome is thought to be typified firstly by demyelination and secondly progressive axonal loss. [40,42,43,105,154] Patients who present following diagnostic delay have greater axonal loss and worse long-term mobility (see

chapter 2). It is therefore conceivable that measuring axonal nerve damage (in a range of inflammatory neuropathies) could be used as a useful prognostication tool (likely combined with other established indices) to predict the likelihood of long-term functional outcome.

3.1.4.4 Titrating therapies to disease activity

The treatment of chronic inflammatory neuropathies (paraproteinaemic, vasculitic, amyloid, CIDP, POEMS) often require long term therapies that are costly and not without risks. Treatment algorithms are broadly designed around measuring clinical response in order to titrate therapy with the aim of achieving clinical stability or improvement whilst minimising side effects, toxicity and complications.[155,156] Fluid biomarkers may detect neuropathology prior to the manifestation of measurable clinical disease or treatment response. It may be that therapies could be titrated in light of more objective axonal degeneration or demyelination as opposed to current methods.

One example of this would be for CIDP, in which cellular and humoral components of the immune system attack myelinated large fibres leading to demyelination. As the disease progresses, axonal loss occurs, which is associated with poor prognosis. [153,157,158] Intravenous immunoglobulin (IVIG), steroids and plasma exchange are key treatment options in CIDP, as are immunomodulatory medications. [159] IVIG is costly at £42.50 per gram, [160] in limited resource, in most cases requires day case hospital stays and is not without complications. For such reasons, significant work has been undertaken to rationalise the use of IVIG in CIDP and related disorders to optimise

dosing, timing intervals and cessation trials. [155] Titrating IVIG to fluid biomarkers of demyelination and axonal loss may be a more precise therapeutic strategy, limiting IVIG and providing patients with optimum therapeutic effect whilst minimising side effects.

Regarding POEMS syndrome, immunomodulatory medication including lenalidomide and thalidomide, and proteasome inhibition with bortezomib and carfilzomib are agents used primarily for the treatment of non-ASCT fit patients. [46] Such agents are potentially neurotoxic and result in sensory, often painful neuropathy in a dose dependent fashion. [161–164] Titration of such medications through monitoring peripheral nerve damage biomarkers may enable more accurate titration to minimise toxicity whilst delivering the highest tolerated dose of drug.

3.1.5 Development of novel biomarkers of peripheral nerve disease

A highly sensitive biomarker assay was developed for the peripheral nerve axonal protein peripherin. The decision to study this protein was from pre-existing knowledge and experience in the success of measuring neurofilament proteins, identification of abundant and highly functional axonal proteins and the specificity of this protein to key structures of the peripheral nerve. The following section provides further rationale behind studying this biomarker in turn, followed by the process of investigation which led to development of a highly sensitive immunoassay through to testing clinical samples.

3.1.6 Intermediate filament proteins; distribution and physiological function

Three types of filament proteins compose the cellular cytoskeleton of peripheral nerves: microtubules, microfilaments and intermediate filaments. [165] Microtubules are made of tubulin and are involved in maintaining cell shape. Microfilaments are mostly made up of actin, responsible for cell movements, muscle contraction, mechanical strength and axonal growth. The organisation of intermediate filaments and their association with the plasma membrane suggest their principal function is to reinforce cells which sustain mechanical stress. Intermediate filaments are classified into five different sub-types (I-V), detailed below (see table 3.1).

Table 3.1: Intermediate filament proteins, molecular weight and tissue distribution. Taken from Lodish et al; Molecular cell biology. [165]

Intermediate filament protein	Molecular weight (kDa)	Tissue distribution
Type I		
Acidic keratins	40-57	Epithelia
Type II		
Basic keratins	53-67	Epithelia
Type III		
Vimentin	57	Mesenchyme
Desmin	53	Muscle
GFAP	50	Glial cells and astrocytes
Peripherin	58	Peripheral and central neurones
Type IV		
Nf-L	62	Mature neurones
Nf-M	102	Mature neurones
Nf-H	110	Mature neurones
Internexin	66	Developing CNS
Type V		
Lamin A	70	Nucleus of all cells
Lamin B	67	
Lamin C	67	

Type I and II intermediate filaments are acidic and basic keratins expressed in epithelial cells. The keratins are the most diverse class of intermediate filament proteins and have several isoforms. About 10 keratins are specific to hard epithelial tissues, and give rise to nails, hair and wool (in animals).

Type III intermediate filaments are vimentin, desmin, GFAP and peripherin. These filaments can form both homo and heteropolymeric filaments. Vimentin is the most widely distributed and supports cell membranes to keep the nucleus and other organelles in place. The other intermediate filaments have much more limited distribution. Desmin filaments are responsible for sarcomere stability in contracting muscle. GFAP forms filaments in glial cells. Peripherin is found in neurones of the PNS, but little is known about its function (detailed later).

The core of axons are filled with type IV intermediate neurofilaments called neurofilaments. Neurofilaments are heteropolymers of three type IV polypeptides; Nf-light (NfL), Nf- medium (NfM) and Nf-heavy (NfH), classified by molecular weight. Microtubules direct the elongation of axons. In contrast, neurofilaments are responsible for radial growth. [166]

Type V intermediate filament proteins are called lamins and are found exclusively in the nuclei of all cells, forming a fibrous network that supports the nuclear membrane.

3.1.7 Peripherin

Peripherin is a type III intermediate filament protein of three major isoforms, 56kDa, 58kDa and 61kDa. Similar to other type III intermediate filaments, peripherin is capable of self-assembly to form homopolymeric filament networks. [167–169] Peripherin can also co-assemble with the neurofilament triplet NFL, NFM and NFH. The first description of peripherin in 1988 identified intense labelling in small calibre nerve fibres in the sciatic nerve and spinal dorsal roots, as well as dorsal columns and dorsal root ganglion on immunocolocalisation studies of rats. [170] In the brain, labelling of components of cranial nerves is seen, as well as thin fibres in specific limited areas including the cerebellum and corticospinal tract of the brain stem. However, the distribution and abundance of peripherin is thought to be primarily in the peripheral axons, and identical to the neurofilament triplet proteins in sciatic axons. [171] The physiological function of peripherin is unknown. Expression is upregulated in different neuronal types following injury, including dorsal root ganglion and motor neurones following sciatic nerve, cortical and thalamic trauma. [172] Such upregulation would suggest peripherin has a role in neuronal regeneration. Genetic mutations of peripherin, NFL and NFH are mostly associated with primary peripheral nerve disorders such as CMT and ALS. Gene mutations in alpha-internexin, a CNS derived neurofilament protein for example are associated with forms of frontotemporal dementia. [173] Thus the association of particular neurofilament subsets with peripheral or central disease match the relative abundance of the subunit in the nervous system.[174]

Peripherin gene mutations in cases of ALS have led researchers to consider the relevance of peripherin in peripheral nerve diseases through disruption of neurofilament networks. [171,175,176] A pathological hallmark of amyotrophic lateral sclerosis (ALS) is of axonal spheroids and perikaryal accumulations of intermediate filament proteins, the neurofilaments and peripherin. [167] It is unknown whether this is a pathological neurotoxic hallmark of disease or a secondary regenerative response.

The relative specificity of peripherin to peripheral nerves coupled with its similar abundance to NFL makes peripherin a promising biomarker candidate for peripheral nerve axonal damage. Being able to identify patients with peripheral nerve axonal damage will assist with diagnosis, neuropathy classification and may also contribute towards our understanding of pathogenesis. Comparing peripherin levels to neurofilament light would enable better understanding of the proportion of neurological damage which is peripheral vs central in mixed disease such as amyotrophic lateral sclerosis. A number of hypotheses and aims were therefore defined with regards to the creation of an immunoassay for peripherin:

3.1.8 Hypotheses

1. Peripherin is present in peripheral nerve axons and is released upon damage
2. Peripherin can be detected and measured reliably using highly sensitive immunoassays
3. Peripherin release is in proportion to peripheral nerve axonal damage/loss
4. Peripherin is not released into to the circulation from central nervous system pathology

3.1.9 Aims

1. Identify the optimum recombinant protein, capture and detector antibody to be used in solid phase immunoassay.
2. Develop an immunoassay to detect peripherin in serum samples at an appropriate level of sensitivity.
3. Identify whether peripherin levels differ between peripheral and central nervous system axonal damage.
4. Define whether there is an association between NFL and peripherin release in peripheral neuropathy.
5. Establish whether levels of peripherin correlate with disease severity

3.2 Identifying the optimum recombinant protein, capture and detector antibodies to be used in solid phase immunoassay.

There are no published data on assays to detect peripherin in the literature. We started by identifying the most appropriate antibodies and recombinant protein, exploring on ELISA and then optimising for sensitivity. A number of preliminary experiments were performed to select an appropriate antibody pair and representative recombinant protein.

3.2.1 Methods

3.2.1.1 Antibody and recombinant selection

The following antibodies were purchased based on the following features:

i. Antibody clone type: either monoclonal or polyclonal antibodies, with a preference for monoclonal antibodies for specificity and batch-to-batch stability.
ii. Antibody purity: affinity purified unconjugated antibodies in PBS were preferred for potential later use with the SiMOA. Highly sensitive immunoassays rely often on binding capture antibodies to beads and pre-labelling of detection antibodies to amplify signal. Impure antibody solutions are prone to nonspecific binding.[146]
iii. Applications: ELISA tested.

Table 3.2: Recombinant proteins and antibodies selected for peripherin assay development

Product	Company	Detail
Recombinant protein #1	Abcam	36kDa fragment, amino acids (AA) 374-470
Recombinant protein #2	Novusbio	80kDa full length with GST tag at N terminal
Recombinant protein #3	Origene	58kDa full length with C-terminal DDK tag

Antibodies	Code	Company	Host	Purity	Immunogen
Monoclonals	MC1	Thermofisher	M	TC	Recombinant full length rat peripherin
	MC2	Abcam	M	TC	Recombinant full length rat peripherin
	MC3	Santa Cruz	M	AP in PBS	Raised against AA 21-90 of human peripherin protein
	MC4	Origene	M	AP in PBS	Full length recombinant human peripherin
	MC5	Novusbio	M	AP in PBS	Full length recombinant human peripherin
	MC6	Sigma	M	AP	Full length recombinant rat peripherin
	MC7	Novusbio	M	AP	AA 374-470

Antibodies	Code	Company	Host	Purity	Immunogen
Polyclonals	PC1	Thermofisher	Rab	Serum	Full length recombinant rat peripherin
	PC2	Abcam	Rab	Serum	Full length recombinant rat peripherin
	PC3	Novusbio	Rab	Serum	Full length recombinant rat peripherin
	PC4	Abcam	Rab	AP	Recombinant mouse peripherin AA 50-150

Antibodies	Code	Company	Host	Purity	Immunogen
Secondary HRP conjugated antibodies (ELISA)	SAR	Dako	Swine	AP	Rabbit IgG
	RAM	Dako	Rabbit	AP	Mouse IgG

M= Mouse, Rab= Rabbit, SAR= Swine anti-rabbit, RAM= Rabbit anti-mouse, HRP=horseradish peroxidase, TC= tissue culture, AP= affinity purified, PBS= phosphate buffered saline

3.2.1.1.1 Recombinant mapping

Mapping the peripherin protein primary amino acid sequence enables visualisation of antibody epitope binding sites of the full-length protein and recombinant protein fragments. This is fundamental to deciding which combination of recombinant, capture

and detection antibody to use. If the only available recombinant protein is a very short length fragment at the C-terminal of the full-length protein sequence, and all available antibodies bind to epitopes at the N-terminus then antibodies will not be able to recognise the recombinant protein. Another consideration is whether the capture and detection antibodies bind to exactly the same epitope which risks competitive binding interactions. [177]

3.2.1.2 Tissue homogenisation of neuronal structures and other organs

Male or female Sprague Dawley adult rats were provided by the animal facility at the Institute of Neurology. All rats were culled by rising carbon dioxide concentration followed by cervical dislocation. All animal procedures were in accordance with the Home Office Guidance on the Operation of Animal Act 1986. [178]

Sciatic nerves were dissected following a protocol adapted from published methods[179] Full details of the materials and methods are included in appendix 2. In short, rats were pinned to a pin board and washed with isoflurane and ethanol sprays. An inferolateral incision to the thigh was made to identify the sciatic nerve from the spine to the posterior fossae of the knee and separate it from the surrounding tissue. The sciatic nerve was cut at the proximal and distal sites, washed three times in Tris buffered saline (TBS) and frozen at -80 ^{o}C in 2 ml Eppendorf tubes. Rat brains and spinal cords were dissected according to previous protocols. [180] The heart, lungs, spleen, kidneys, liver and pancreas were also all dissected using anatomical guides. [181]

The tissue homogenisation protocol was adapted from a published protocol. [182] Tissue was transferred into a 50 ml Falcon tube. Ice cold tris buffered saline (TBS) was added to each tissue at 1ul per ug of tissue with a protease inhibitor cocktail (Thermofisher) at 5ul per 100ug tissue. Tissues were homogenised with a mechanical homogeniser for 10 minutes until the tissue was broken down in solution. Homogenate was left on ice for 30 minutes, then centrifuged at 12,000 g for 30 minutes at 4 degrees. The supernatant was aspirated and placed in a fresh 2ml Eppendorf tube. Protein concentration was ascertained through a bicinchoninic acid (BCA) assay (Thermofisher) before the recovered tissue extract was frozen at -80 ^{o}C.

3.2.1.3 Optimising lysis buffers

Lysis buffers were tested to ascertain whether proteins could be enriched dependent on their cellular location or solubility, and also to determine whether non denaturing buffers could be used, which otherwise would potentially denature antibodies if present in the homogenate for testing immunoassays. Buffers from four previously published protocols designed to optimise cytoskeletal protein lysates from neuronal tissues were selected for use. [183–186] Details of the buffers are in appendix 3. In short, mild 'neuronal protein' extraction buffer (buffer A) was added to rat tissue when mechanically homogenising using the same methodology in the "tissue homogenisation of neuronal structures" section above. Soluble protein supernatant was separated and the insoluble protein pellet was further dissolved by a zwitterionic buffer (buffer B), centrifuged and supernatant removed, before dissolving the final pellet with ionic detergent (buffer C) for insoluble fractions. Each tissue lysate was then tested for peripherin using western blotting method. Tissue lysate loading concentration was standardised to 30 ug per

lane, using monoclonal #3 as the primary antibody and rabbit anti-mouse light chain specific HRP conjugate as secondary. A heavy chain secondary antibody could demonstrate signal at 50kDa through cross reactivity to rat IgG and could overwrite the peripherin band hence the use of the light chain specific secondary.

3.2.1.4 Dot blot

Full details of the materials and methods are included in appendix 2. 2 ul of 1 ug/ul sciatic nerve homogenate or recombinant peripherin protein were pipetted as a drop to a nitrocellulose membrane and allowed to dry for 30 minutes. A second 2 ul drop of sciatic nerve homogenate buffer only was made 2 cm below and allowed to dry. Membranes were blocked using TBS in 5% Bovine Serum Albumin (BSA) for 1 hour. Membranes were soaked in primary antibody solution (monoclonal and polyclonal antibodies to peripherin) at 1:1000 dilution in TBS 1% BSA. Membranes were washed with TBS 0.05% tween-20 for 5 minutes three times. Membranes were then soaked in HRP-conjugated anti-species antibody (Dako) at 1:5000 dilution in TBS 5% BSA for 30 minutes. Membranes were again washed three times before electrochemiluminescent (ECL) reagent (Thermofisher) was applied and imaged immediately using a ECL camera (BioRad).

3.2.1.5 Western blot

Western blots against whole nerve tissue were performed to clarify the specificity of peripherin antibody binding. Full details of the materials and methods are included in appendix 2. Protein loading was standardised to 0.1 ug for recombinant proteins and 30

ug for tissue homogenates with 30 ul sample loaded. Proteins were electrophoresed through Nupage 4-12%, 1 mm 10 well gels at 200v for 45 minutes. Proteins were transferred to nitrocellulose membranes, blocked for 30 minutes with TBS 5% BSA. Anti-peripherin primary antibody at 1:1000 in TBS 1% BSA were applied overnight at 4 degrees. Membranes were washed five times for five minutes with TBS tween 0.1%. Membranes were immunoblotted with HRP-conjugated anti-species secondary antibodies at 1:5000 with TBS 1% BSA, washed once more, before the ECL reagent was added. Membranes were imaged immediately with a Bio-Rad ECL image analyser or Alpha Innotech FluorChem SP Gel Imaging System.

3.2.1.6 Immunoprecipitation

One consideration was that antibody epitopes for peripherin reside within the 3D confirmation of the endogenous folded protein, where the process of denaturing and linearising proteins on western blot enabled detection through immunoblotting. An immunoprecipitation method was therefore opted for.

Non-ionic detergents were used in the tissue buffer so proteins were not denatured or linearised before antibodies were incubated. A band detected on western blot would therefore signify the antibody has effectively isolated the protein of interest in its non-denatured, endogenous state.

Sciatic nerve homogenisation was performed as previously described using non-denaturing immunoprecipitation buffer (see appendix 3). Four separate reaction

variables were set up as per table below in table 3.3. Lysate protein load was standardised to 0.7 mg per reaction with 2ug antibody made up to 1000ul total volume. Each reaction was incubated overnight at 4 degrees. 20ul of protein A bead slurry was added to each reaction and incubated for two hours at 4 degrees. Samples were spun at 0.5rcf for three minutes and the flow through separated. Beads were washed three times with immunoprecipitation buffer before all buffer was aspirated and removed. Laemmli buffer and 10% BME were added to the beads, boiled at 65 degrees and centrifuged. The supernatant was used for western blotting. This was compared against non-immunoprecipitated sciatic nerve lysate (the 'input' lane) as a reference.

Table 3.3: Immunoprecipitation reaction types

Variables	Lysate	Antibody
Reaction 1	✓	✓
Reaction 2	✓	Normal healthy control (normal serum igG from host lysate species)
Reaction 3	✓	×
Reaction 4	×	✓

3.2.2 Results

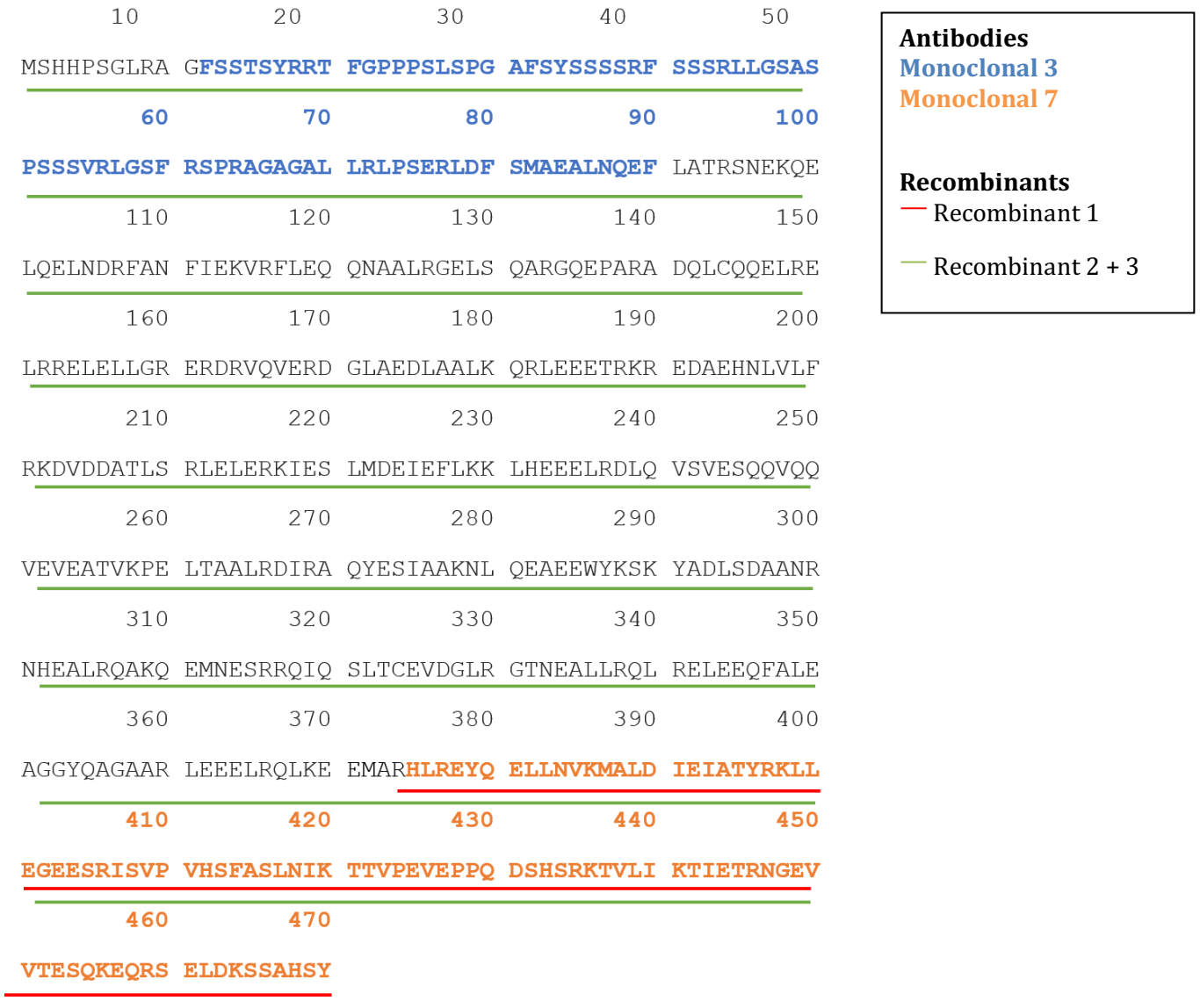
3.2.2.1 Recombinant mapping

The first recombinant protein was a short N-terminal fragment of peripherin, and although monoclonal antibody 7 (MC7) would bind to this structure, it could bind to the whole fragment and this might prevent detector antibody binding. This would likely be the case with the other antibodies, and thus recombinant 1 was rejected from further use. A number of preliminary tests were performed using recombinant protein 1 which failed, and this is the likely explanation.

We tested each antibodies' ability to detect peripherin from a sciatic nerve tissue homogenate as this would contain full length endogenous peripherin. Full length native peripherin should enter the circulation in axonal damage. Post-translational

modifications and recombinant protein tags could influence protein folding and thus alter epitope binding sites and influence detection.

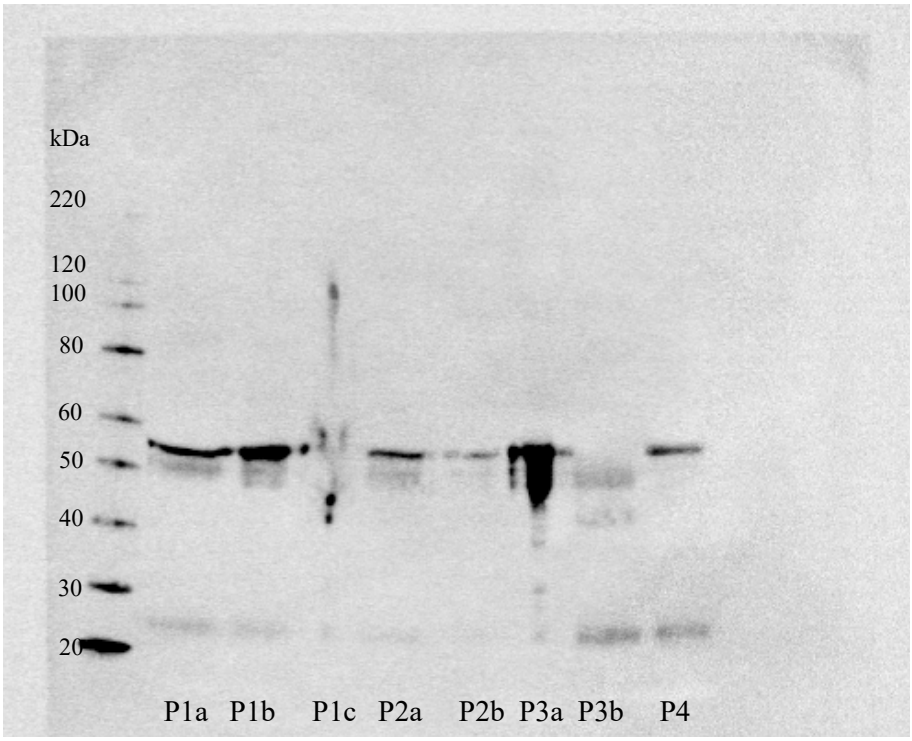
Figure 3.4: Peripherin protein isoform 1 full length amino acid sequence, antibody and recombinant protein map.



3.2.2.2 Optimising lysis buffers

Buffers from each protocol sufficiently solubilised peripherin. Neuronal protein extraction buffers using mild detergent (P1a, P2a, P3a, P4- see appendix 3) effectively solubilised peripherin. Ionic denaturing detergents (P1b, P1c, P2b, P3b- see appendix 3) are not necessary to solubilise peripherin protein from sciatic nerve homogenates. This was useful going forward using ELISA immunoassays where initial testing using tissue homogenate was relied upon to test antibody combinations. For ease, tissue homogenisation was subsequently performed in TBS only.

Figure 3.5: Western blot of sciatic nerve lysate with monoclonal #3 comparing different lysis buffers.

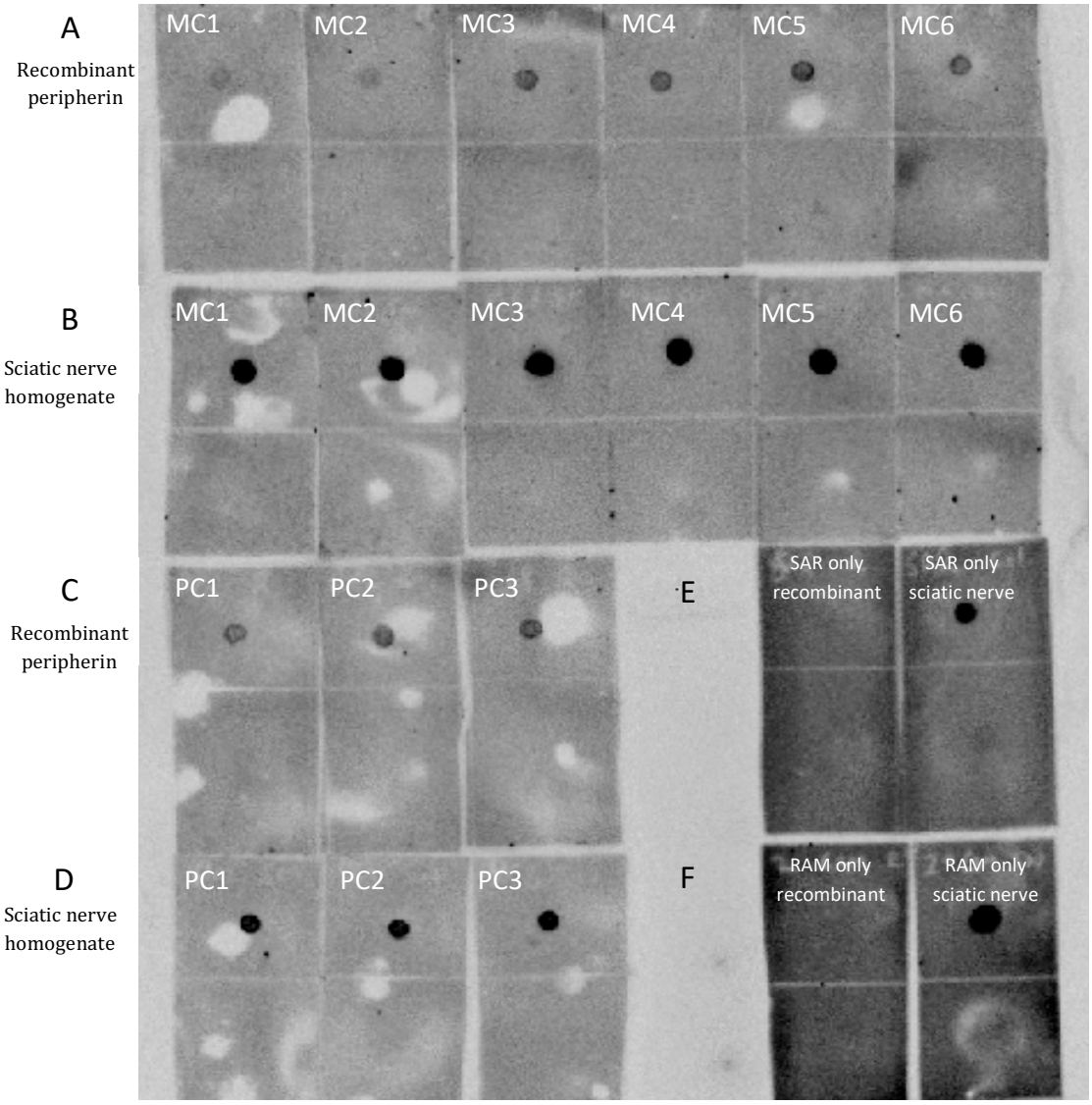


P= tissue lysis protocol 1 vs 2 vs 3 vs 4. Note only protocol 1 had 3 different buffers (a, b, c) compared to protocol 2 and 3 which had 2, and protocol 4 using a neuronal protein extraction buffer (Thermofisher).

3.2.2.3 Dot blot experiments

Dot blot experiments using both recombinant purified peripherin #2 (A) and sciatic nerve homogenate (B), probed for with monoclonal antibodies to peripherin MC1-MC6 demonstrate clear signal produced across all antibodies thus signifying likely binding to peripherin (see figure 3.6). Signal was more pronounced in sciatic nerve homogenates compared to that of the recombinant. The actual concentration of peripherin in the sciatic nerve tissue is unknown which likely explains this discrepancy. This was replicated with polyclonal antibodies PC1-PC3 (C and D). No non-specific binding using recombinant protein was seen with E +F (secondary HRP conjugated antibodies), however there was binding in both instances to the sciatic nerve tissue indicating a degree of non-specific binding to sciatic nerve proteins/immunoglobulin. This was an important consideration in developing the immunoassay as non-specific secondary antibody binding to sciatic nerve proteins could produce false positive results.

Figure 3.6: Dot blot experiment demonstrating binding of antibodies to target.



Monoclonal antibodies (numbered 1-5 as per table 3.2) are demonstrated to bind to recombinant purified peripherin (A) and sciatic nerve homogenate (B). Note lower segment is distilled water only and hence no binding. Polyclonal antibodies (1-3 as per table 3.2) bind with similar affinity to recombinant (C) and sciatic nerve (D). Secondary HRP conjugated antibodies only (E and F) bind to sciatic nerve tissue homogenates (right column) but not recombinant protein (left column).

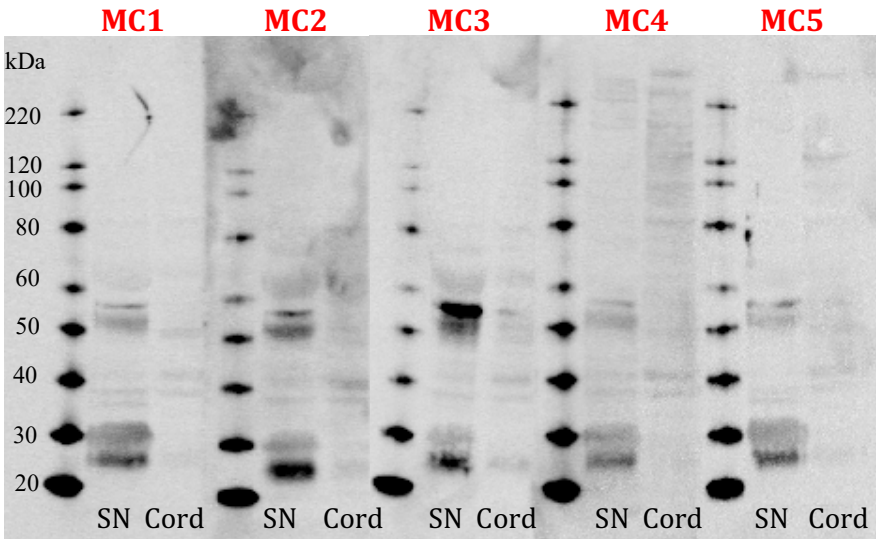
3.2.2.4 Western blot experiments

In figure 3.7a, sciatic nerve homogenate was compared to spinal cord. Monoclonal antibodies all show specific and consistent band at 58kDa representing peripherin.[171] There appears to be a double band, which may represent the two peripherin isoforms of 56kDa and 58kDa. This is not seen in the cord homogenate. Monoclonal 3 appears to have the greatest affinity to bind peripherin. A strong band is seen at 25kDa likely representing cross reactivity of the secondary rabbit anti-mouse HRP conjugated antibody with tissue homogenate immunoglobulin light chains.

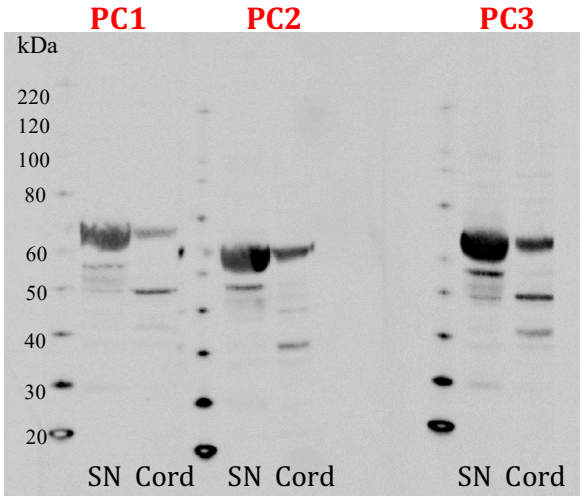
Similar results are found in C where brain homogenate was then compared to sciatic nerve and recombinant protein #2 (monoclonal 5 only). A very clear solitary band is present at 58kDa in the nerve, as is a band detecting recombinant peripherin protein #2 by monoclonal antibody 5, which is 80kDa due to the addition of a GST tag. There is a very similar band pattern for each polyclonal (B), which differs from the monoclonals. The polyclonals clearly detect the 58kDa band of peripherin in the sciatic nerve and not in the cord. This may be a result of differing peripherin content in the sciatic nerve compared to the spinal cord, in which peripherin is likely to be released in small amounts due to the presence of the neuronal root and anterior horn cells only in the cord compared to being present throughout the sciatic nerve. They all also demonstrate clear binding with an intense thick band at approximately 61-65kDa, which could represent the known peripherin isoform of 61kDa, or could be due to non-specific binding since this is not seen with the monoclonals. A band is seen at 50kDa which represents anti-species HRP conjugated antibody cross reacting with tissue homogenate immunoglobulin heavy chain.

Figure 3.7: Western blot analysis of peripherin detection in rat sciatic nerve vs spinal cord tissue and in rat sciatic nerve compared to brain.

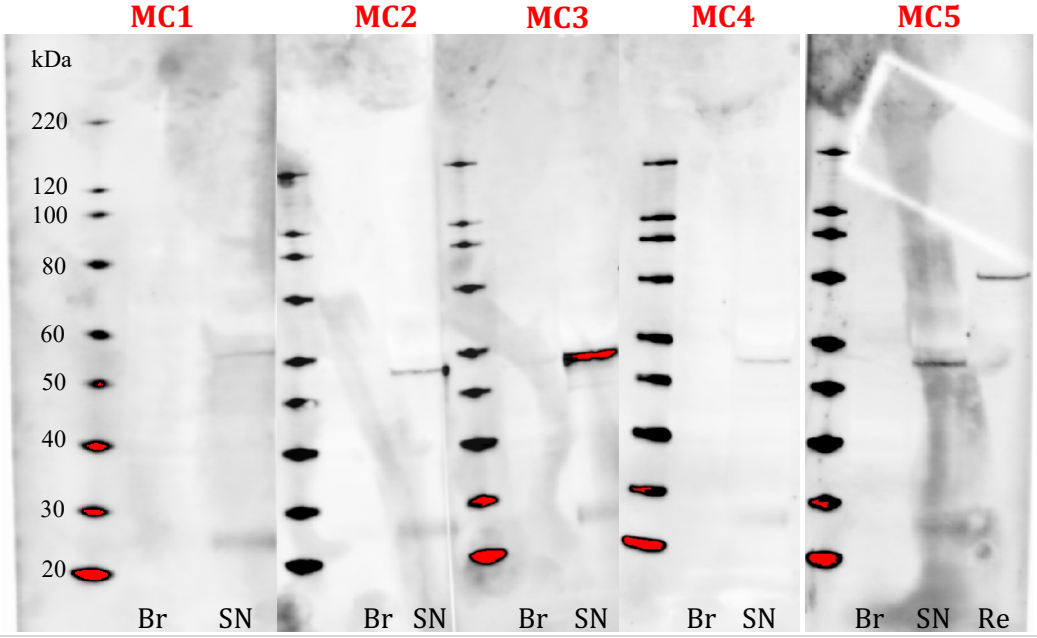
A



B



C



(A) using monoclonal antibodies (MC1-5 as per table 3.2) comparing sciatic nerve to cord homogenates, then using polyclonal antibodies (B). Lastly, comparing brain to sciatic nerve homogenates using monoclonal antibodies MC1-MC5, including to recombinant peripherin protein with GST tag, molecular weight 81kDa using monoclonal 5. Note membranes were cut to bathe in separate mono and polyclonal antibody, thus align differently, but have own molecular weight ladder marker to refer to.

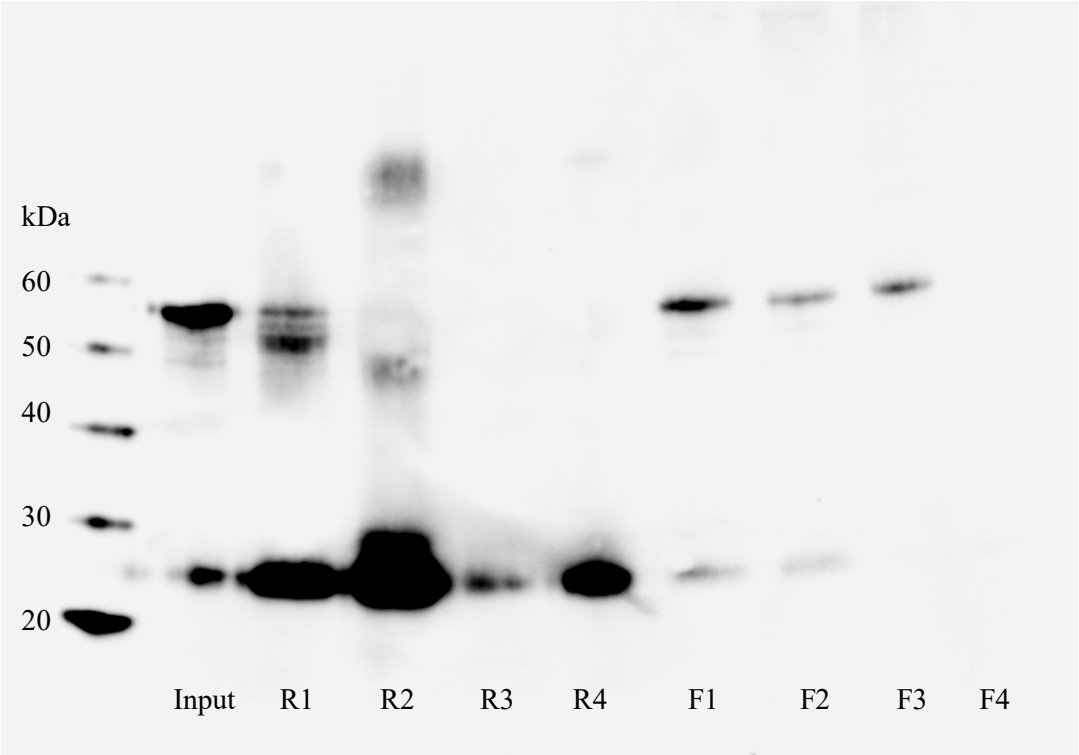
Immunoprecipitation

The input lane serves as a reference demonstrates peripherin detection at 58kDa, possibly in a double band with its isoform. Reaction 1 demonstrates anti-peripherin antibody has bound and isolated peripherin from the sciatic nerve lysate in its endogenous form without denaturing agents. Control lanes (R2-4) do not show bands at 58kDa. Flow through 1 (F1) represents flow through of R1 and demonstrates excess peripherin that was not isolated by the anti-peripherin antibody, and F2, F3 demonstrate samples in R2,R3 contained peripherin but were not effectively isolated in R2 and R3 due to lack of anti-peripherin antibody.

This experiment demonstrates anti-peripherin antibody is able to bind peripherin antigen in its endogenous, tertiary 3D structure without requiring strong detergents to linearise the protein.

Figure 3.8: Immunoprecipitation of rat sciatic nerve with anti-peripherin monoclonal antibody and light chain specific secondary HRP conjugate.

Variables	Lysate	Antibody
Reaction 1	✓	✓
Reaction 2	✓	Normal healthy control (normal serum igG from host lysate species)
Reaction 3	✓	×
Reaction 4	×	✓



**R= reaction, F= flow through*

3.3 Development of ELISA to detect peripherin

The experimental variables below were systematically addressed, tested, and optimised. Once a sandwich ELISA had been developed and optimised, the sensitivity of this assay was tested. Without knowing the normal levels of peripherin in the blood, an optimum sensitivity was indeterminate. Neurofilament light should be of similar abundance to peripherin, [171] and normal levels of neurofilament light are around 20-40pg/ml (and correlate with age).[140] We therefore opted for a limit of detection under 20pg/ml as being a necessary target and broadly acceptable.

3.3.1 Methods

Firstly, an indirect ELISA technique was used to bind recombinant protein or sciatic nerve homogenate to the plate and assess each antibody's ability to bind to the target antigen confirming the antibodies functionality. Once achieved, different combinations of antibodies in a sandwich ELISA were trialled, and the following variables systematically experimented upon to develop the immunoassay and optimise for the best signal to noise ratio.

Experimental variables:

Plate type

Comparing plate surface treatment.

- Nunc-immuno Polysorp (Sigma)- high affinity to hydrophobic molecules
- Nunc-immuno Maxisorp (Sigma)- high affinity to mixed hydrophobic/philic domains
- Nunc-immuno Multisorp (Sigma)- high affinity to hydrophilic molecules

Blocking solution

- TBS or PBS in 5% BSA or 5% skim milk + 0.05 % Tween 20

Buffers

- Calcium carbonate, immunoprecipitation buffer, tissue lysis buffers 1-4, barbitone buffer, TBS, PBS (see appendix 3 for buffer compositions)

Wash

- TBS or PBS with 0.05% Tween 20

Antibody diluent

- Same as blocking solution but 1% protein and no detergent

Capture antibody

- Using both monoclonals and polyclonals
- Different concentrations using chequerboard titrations

Standard

- Using recombinants 1-3 and sciatic nerve homogenate
- Using different concentrations starting at 1:1000 in chequerboard titration

Detector antibody

- Using monoclonals and polyclonals (different species to capture antibody)
- Different concentrations using chequerboard titration.

HRP conjugated secondary anti-species antibody

- Different concentrations of rabbit anti-mouse or swine anti-rabbit HRP conjugated antibodies (Dako) using chequerboard titrations.

The final ELISA method is detailed below.

Monoclonal 3 capture antibody was diluted with calcium carbonate to 1:3000 and 100 ul was added per well and left overnight at 4 ^{o}C. Capture antibody was discarded and 100 ul of 5% skim milk in TBST 0.05% Tween 20 blocking solution was added and incubated by shaking for one hour at room temp. The plate was then washed three times with 200 ul TBST 0.05% Tween 20 wash solution before blotting to dry. Recombinant protein was diluted at 1/500 to 340,000 pg/ml for the top standard and a double dilution performed to 10.3 pg/ml to determine the sensitivity of the assay, added at 100 ul per well in duplicate and incubated for 1.5 hours at room temperature shaking at 600 rpm. The plate was washed three times again and blotted dry, and detector PC1 antibody was diluted to 1:2000 using TBS 1% skimmed milk, applied at 100 ul per well and incubated for one hour at room temperature shaking at 600 rpm. The plate was washed three times and blotted dry before secondary swine anti-rabbit HRP conjugated antibody was added at 1:5000, 100 ul per well, incubated for one hour at room temperature shaking at 600 rpm. The plate was washed three times and blotted dry before 100 ul of 3,3'3,3'- Tetramethylbenzidine (TMB- Thermofisher) was applied for 15 minutes in the dark before 100 ul of one molar sulfuric acid was applied to each well and absorbance read at 450 nm on a plate reader.

3.3.2 Results

Figure 3.9 demonstrates the efficacy of each monoclonal antibody in turn to detect peripherin. Monoclonal antibody 6 clearly outcompetes any of the other antibodies in terms of signal production, with saturation reached at 80,000 pg/ml and a signal of

approximately 1 OD. Monoclonal 3 was deemed the second most effective, and had not reached saturation at the top calibrator (340,000 pg/ml). Note that changing polyclonal antibody as detector did not appear to significantly change signal (between graphs A-C). Monoclonal 6 capture and polyclonal 1 was then used in the final sandwich ELISA format to ascertain sensitivity for peripherin detection. Signal begins to plateau at the top standard of 340,000 pg/ml. The lowest detectable signal change was at a concentration of 332 pg/ml, with a signal to noise ratio of 1.3 and coefficient of variance (CV) of 1%. Lower limit of detection was 313.5 pg/ml, calculated as the mean of 16 replicated blank values (OD) plus three times the standard deviation. Lower limit of quantification was 1162 pg/ml, calculated as the mean of 16 replicated blank values (OD) plus 10 times the standard deviation.

As explained before, a limit of detection of 20 pg/ml was determined as the desired sensitivity of this assay. Clearly a far greater level of sensitivity was desired. It was therefore decided to transfer the format of the assay from ELISA to SIMOA.

Figure 3.9: Sandwich ELISA of capture monoclonal and polyclonal antibody detectors. A= Polyclonal 1 detector, B= Polyclonal 2 detector, C= Polyclonal 3 detector.

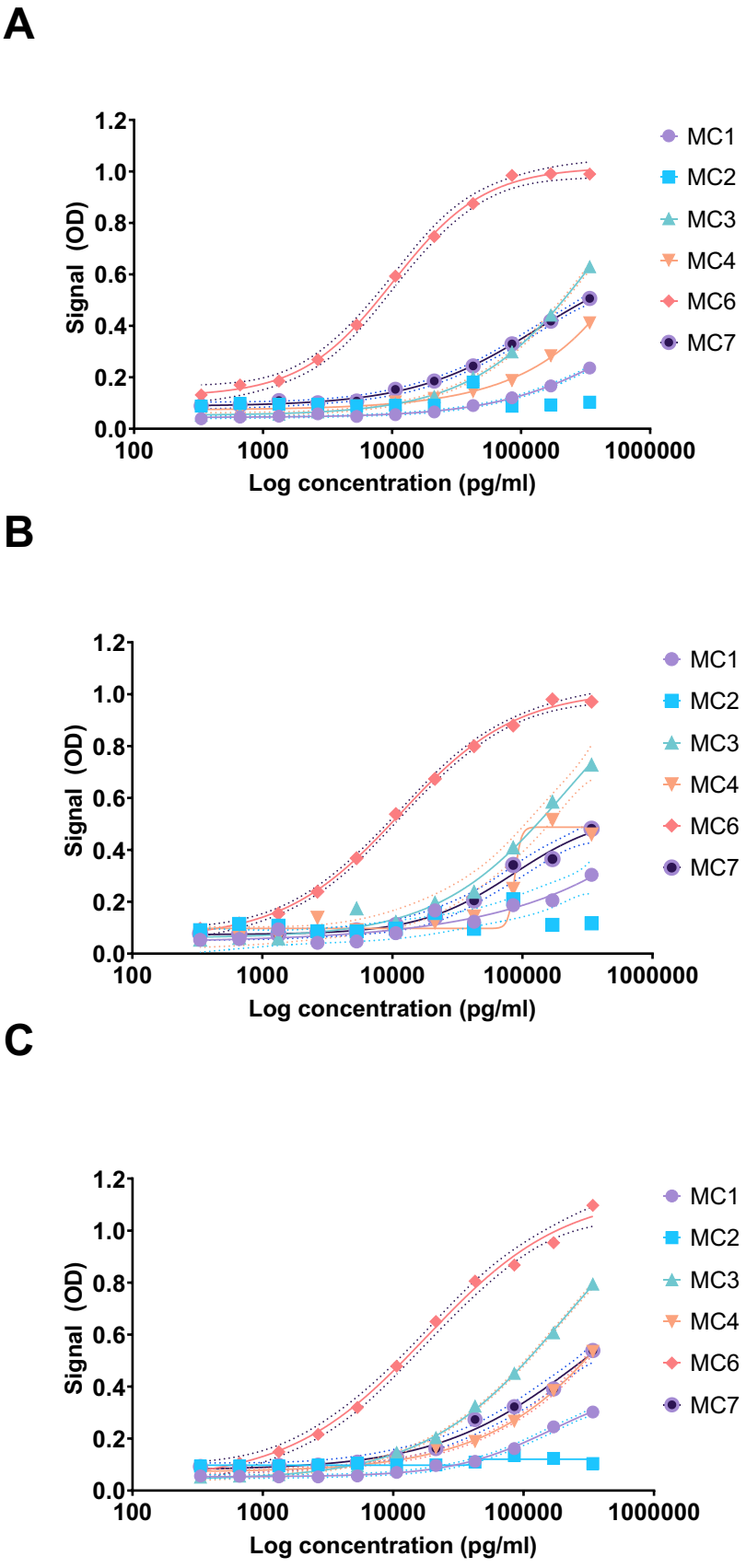
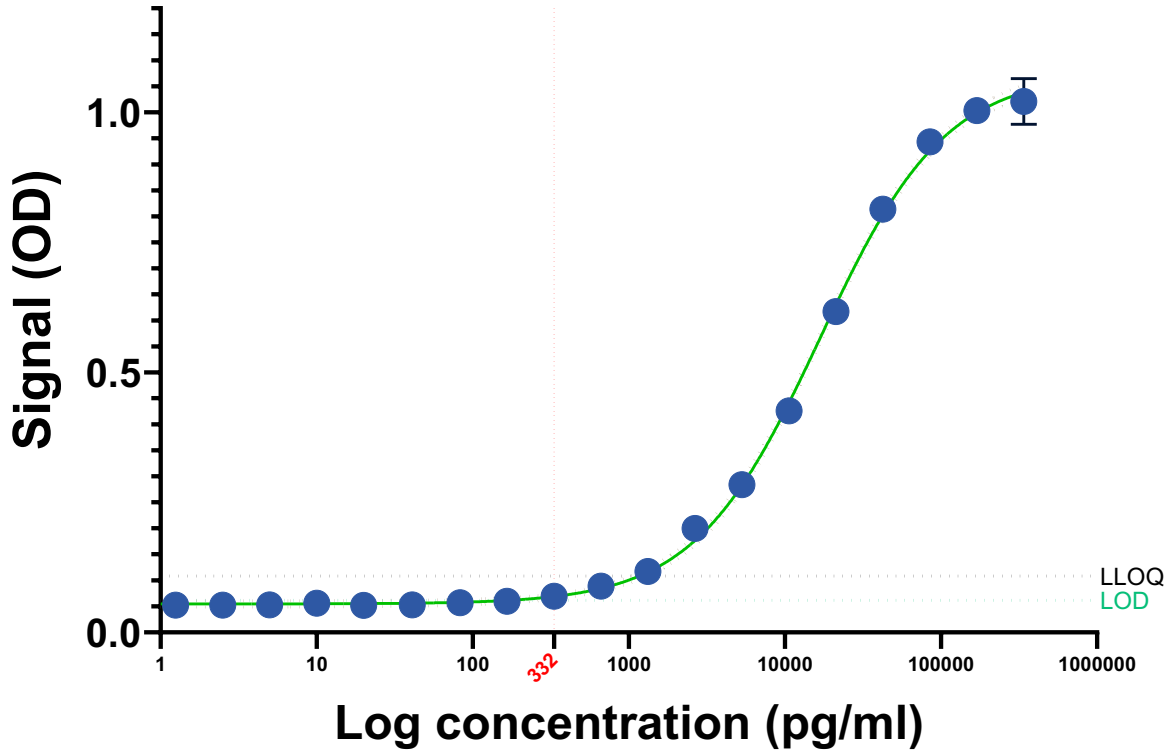


Figure 3.10: Sensitivity of sandwich ELISA to detect recombinant peripherin protein



3.4 Development of SiMOA immunoassay to detect peripherin

Transferring the assay to SIMOA would require further antibody combination testing to select the best pair, followed by optimising reagents to enhance signal to noise ratio, before testing clinical samples and validation.

3.4.1 Standard curve

3.4.1.1 Method

Detailed instructions of the antibody bead coupling and biotinylation can be found in the SiMOA Homebrew Assay Development Guide (Quanterix).[146] In short, capture antibodies were prepared by buffer exchange into the Quanterix recommended bead conjugation buffer using Amicon Ultra-0.5 50kDa centrifugal filters (Merk, Germany) following manufacturer recommendations. Paramagnetic carboxylated beads were washed and prepared to provide a supply of $1.4x10^9$ beads per ml of capture antibody solution. Conjugation of the capture antibodies to beads was based on 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry and performed according to Quanterix® manual protocol, and tested using a range of antibody and EDC concentrations (see below). Beads were placed on a rollator at 2-8^{o}C for 120 minutes (HulaMixer, Thermofisher) to conjugate. Beads were then washed and blocking solution was added and incubated at 2-8^{o}C for 45 minutes. Following three washes, the conjugated beads were suspended and stored at 4^{o}C pending use.

The detection antibodies were buffer exchanged into the biotinylation reaction buffer provided by Quanterix® using Amicon Ultra-0.5 50kDa filters. Antibody concentration was adjusted to various concentrations (between 0.3-2 mg/ml) with biotinylation reaction buffer prior to conjugation to 8.9 mM NHS-PEG4-biotin (Thermofisher) at various ratios (x40 or x60) and incubated for 30 minutes at room temperature. Unreacted material was removed through additional buffer exchange using Amicon filter devices.

Samples were assayed in duplicate on a SiMOA HD-1 instrument (Quanterix) using a 2-step assay protocol which begins with an aspiration of $2x10^7$ beads/ml of capture antibody bound to carboxylated paramagnetic beads followed by the addition of 20ul of biotinylated antibody and 100ul of sample (or calibrator) to the bead pellet. Following a 47-cadence incubation (1 cadence = 45 seconds), the beads were washed, followed by addition of 100 µL streptavidin-conjugated β-galactosidase (Quanterix) (tested at different concentrations to optimise). This was followed by a 7-cadence incubation and a wash. Before reading, 25 µL resorufin β–D-galactopyranoside (RGP- Quanterix) was added.

Each capture antibody was made to test an initial 0.3ml lot of beads with 0.2 mg/ml antibody and 0.3 mg/ml EDC with conjugation performed at 2-8 degrees C. Antibody combinations (capture and detector) were then tested to detect recombinant peripherin protein #3 from a standard curve varying in concentration depending on the requirements of the experiment. The combination with optimum signal:noise was then

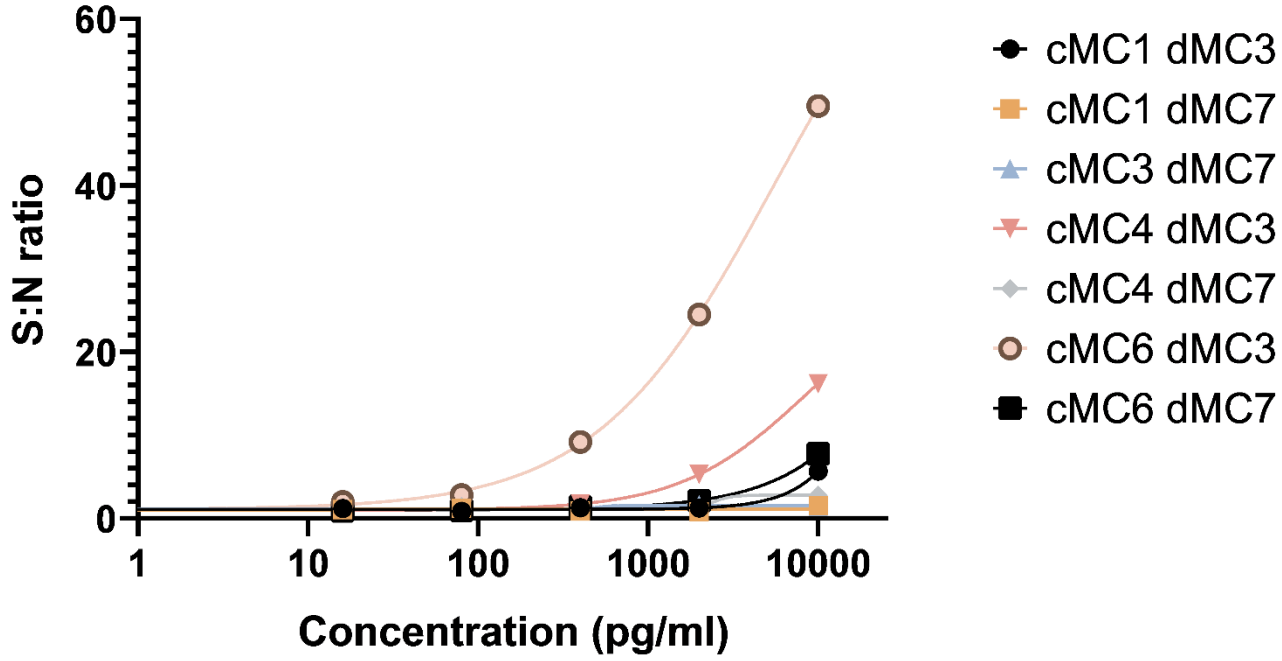
selected, and the following assay variables were optimised for further development in turn in the order below to determine which was best at detecting very low concentrations of peripherin. Following each experiment, the variable with best signal:noise was selected and introduced to the assay methodology, and hence signal to noise improves cumulatively throughout the reagent development steps.

- Two step vs three step assay protocol
- Biotinylation ratio of detector (i.e. x40, x60)
- Capture antibody concentration for bead conjugation (0.1, 0.2, 0.5 mg/ml)
- Concentration of biotinylated antibody (0.3, 1 and 2ug/ml)
- Concentration of SBG (50, 150, 250pm)
- Addition of helper beads

3.4.1.2 Results

Analysing the combination of capture and detector antibodies, it is clear that MC6 capture, MC3 detector has the best signal to noise combination (see figure 3.11), and hence this combination was selected for further optimisation.

Figure 3.11: SIMOA assay comparing signal:noise of monoclonal capture and detector antibody combinations to peripherin recombinant protein



Further optimisation steps were performed as part of the assay development guideline.[146] Assay conditions were tested in turn, with the best assay parameter being selected and then kept constant for subsequent assays (see table 3.4 for details). The optimised assay involved a 2-step protocol, using 60X biotinylation of the detector, at a concentration of 1 ug/ml, with a capture bead concentration of 0.2 ug/ml coating, SBG concentration of 50pM and 350K helper beads per cuvette. The optimised assay standard curve is depicted in figure 3.12. This assay demonstrates low AEB signal coefficients (0.7-5.4%) and error of predicted concentrations (0.6-7.5%). The limit of detection was calculated by calculating 2.5 times the standard deviation plus the mean AEB (signal) of 10 replicate results of the blank calibrator (i.e calibrator A). This was calculated to be 11.3 pg/ ml. The lower limit of quantification (using CV profiling to calculate the lowest concentration at which the CV's are less than the 20% threshold) was 28.5 pg/ml.

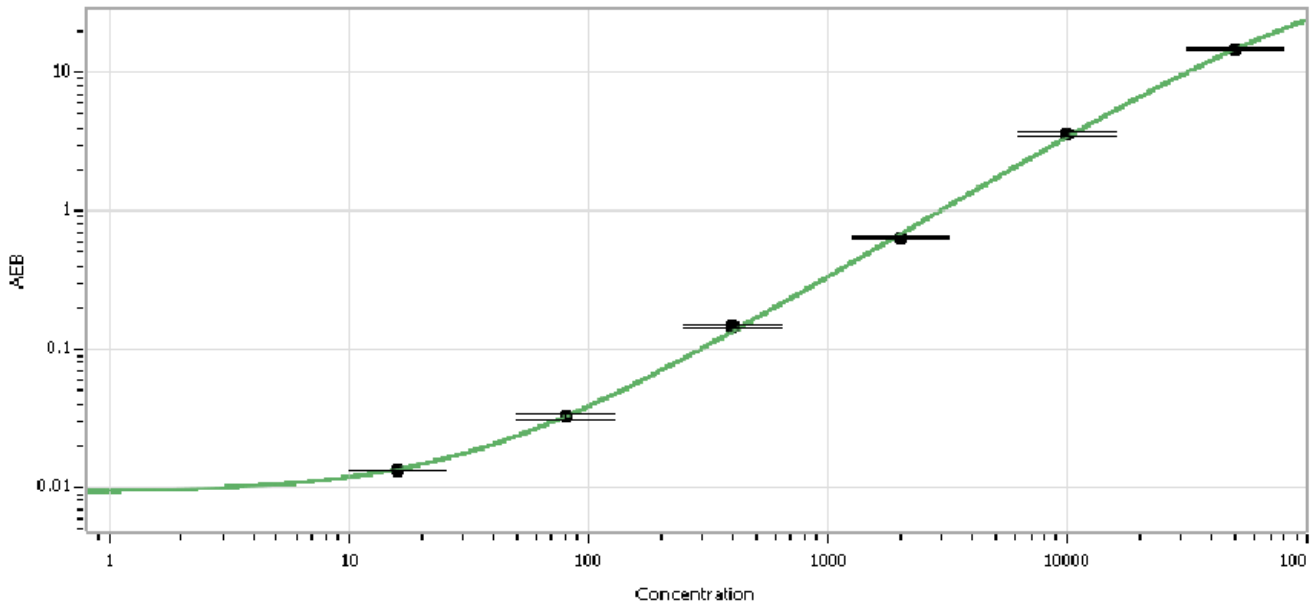
Table 3.4: Optimisation of assay variables using MC6 capture, MC3 detector using SiMOA to improve signal:noise ratio.

Standard curve conc pg/ml	2 step (S:N)	3 step (S:N)	Biotin ratio X40 (S:N)	Biotin ratio X60 (S:N)	Capture conc 0.1 (S:N)	Capture conc 0.2 (S:N)	Capture conc 0.5 (S:N)
0							
7	1.46	1.15	1.46	1.39	1.53	1.60	1.21
10.5	1.77	1.14	1.77	1.61	1.78	1.87	1.39
15.8	2.13	1.10	2.13	2.07	2.08	2.38	1.49
23.3	2.62	1.12	2.62	2.53	2.84	2.84	2.09
35.5	3.21	1.47	3.21	3.24	3.89	3.93	2.65
53.3	4.33	1.58	4.33	4.52	4.44	5.52	3.58
80	5.93	1.99	5.93	6.04	7.38	7.45	4.61
Selected variable	✓			✓		✓	

Standard curve conc pg/ml	Biotin conc 1ug/ml (S:N)	Biotin conc 3.5ug/ml (S:N)	Biotin conc 8.4ug/ml (S:N)	SBG 50pM (S:N)	SBG 150pM (S:N)	SBG 250pM (S:N)	Helper bead 150K (S:N)	Helper bead 250K (S:N)	Helper bead 350K (S:N)
0									
3.2	1.46	1.08	1.08	1.25	1.20	1.13	1.33	1.06	1.69
16	2.12	1.70	1.32	2.13	2.18	1.74	2.63	2.18	3.30
80	6.33	4.01	2.49	6.80	6.12	4.78	8.25	7.83	9.27
400	18.45	12.82	7.06	24.28	18.34	13.21	29.02	26.09	34.36
2000	95.31	82.46	45.07	61.57	34.94	24.85	63.35	56.13	72.27
10000	212.73	192.36	91.92	112.87	262.63	128.17	110.19	92.60	122.82
50000	NR	NR	NR	539.89	244.53	NR	540.08	503.72	634.03
Selected variable	✓			✓					✓

Note the ticks represent the combination selected with the best signal to noise (S:N) which was then incorporated in ongoing experiments as a 'fixed' parameter whilst other variables were altered. As experiments progressed, a larger calibration curve was selected as preliminary tests using samples suggested a far higher concentration of peripherin than originally expected, and thus a larger calibration curve was required.

Figure 3.12: Representative calibration curve of optimised peripherin assay



Average enzyme per bead (AEB) signal on the Y axis and calibration curve concentration on the X. The curve was performed by analysing 5-fold dilutions of peripherin calibrator at 50,000 pg/ml to 80 pg/ml (and blank) in duplicate, and fitting the digital signal on a four-parameter logistic curve (goodness-of-fit=0.99). The AEB signal coefficient in this run was between 0.7% and 5.4% whilst percentage error of predicted concentrations was from -0.6% to 7.5%. Horizontal bar is standard error of test result. Note as experiments progressed, a larger calibration curve was selected as preliminary tests using samples suggested a far higher concentration of peripherin than originally expected, and thus a larger calibration curve was required (hence the lowest test concentration going from 3.2 to 80 pg/ml).

3.4.2 Choice of diluent

3.4.2.1 Method

Seven diluents were selected for study; Diluent A-E from a homebrew diluent kit (Quanterix), Lysate diluent B (Quanterix), and neurofilament diluent (Quanterix). Each was used in turn as diluent for the standard curve and for preliminary spike recovery experimentation to determine the optimal diluent. Pooled healthy control serum samples were diluted 1 in 8 (the MRD) with each diluent to create a 'sample matrix'. For each diluent, 10,000pg/ml of recombinant protein was spiked into the sample matrix, and also into diluent only. A non-spiked sample matrix was also included to measure endogenous peripherin (see formula below).

A diluent which most similarly reflects the matrix of the protein in human serum will likely perform most optimally. Often matrix effects, that is the effect that other substances in the sample may have on the ability to detect the specific target protein, are most commonly observed when using serum or plasma samples. Matrix components can affect the binding of antibody to protein or alter the signal to noise ratio, and are likely attributed to the pH of the sample, high viscosity, direct interaction with the protein of interest or salt concentrations.[187] Samples which exhibit matrix effects will display poor spike recovery, and diluting samples in the appropriate diluent may improve this.

The optimal diluent was determined by first comparing the signal:noise of the spiked sample versus that of the non-spiked, and then secondly from the AEB signal created from the spiked sample matrix compared to that of diluent alone. If significant matrix effects existed, firstly signal:noise would be poor, and secondly, the difference between spiking sample and diluent only would be large. This would result in impaired accuracy of measurement of endogenous samples when comparing against the calibration curve (which is recombinant diluted in diluent alone). To analyse the difference between spikes in sample matrix to that of diluent, the formula below was used, and the diluent with a % relative error (as a measure of accuracy) closest to 100% was selected.

$$\text{RE (\%)} = \frac{\text{Analyte concentration measured in spiked sample- analyte concentration in neat sample}}{\text{Analyte concentration measured in diluent alone}} \text{ x100}$$

3.4.2.2 Results

Table 3.5 summarises the signal to noise ratios of the spiked samples versus that of non-spiked, and secondly the % relative error from spiking sample matrix with 10,000 pg/ml peripherin compared to that in diluent alone. The diluent which performed best in both circumstances was Lysate diluent B, with several other diluents displaying evidence of significant matrix effects, with serum concentrations measured far below that of spiked diluent. Lysate diluent B was then taken forward for subsequent validation testing.

Table 3.5: Results of choice of diluent experiment

Diluent	S:N	% RE
A	22.6	60.0
B	12.4	45.6
C	4.7	26.1
D	24.2	36.0
E	19.9	41.8
NfL	26.5	49
Lysate diluent B	**26.1**	**135**

3.4.3 Parallelism and minimum required dilution

3.4.3.1 Method

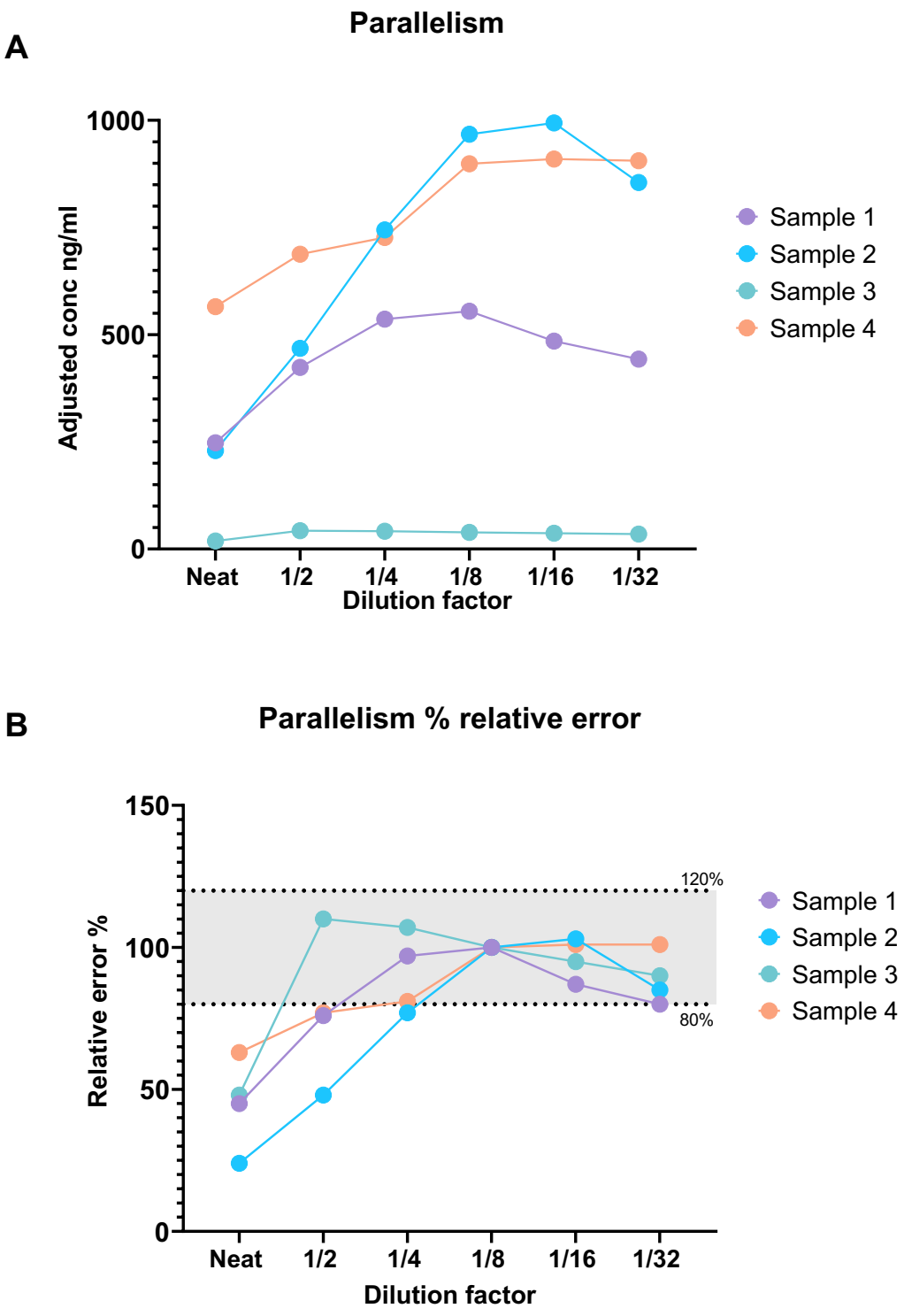
Parallelism was used to determine whether the binding characteristic of the endogenous analyte (peripherin) to the antibodies was the same for the recombinant calibrator. Four samples were identified with high endogenous concentrations of peripherin. Each sample was then serially diluted 1:2 six times. Samples were tested on the same run, in duplicate, and the back calculated concentrations used for analysis. Dilution-adjusted concentrations were plotted to set the minimum required dilution (MRD), and to then determine parallelism. MRD was set at the first dilution in which the remaining samples' dilution-adjusted concentrations were measured at +/- 20 % of the defined MRD result. The % relative error (RE) of each sample was then calculated against the MRD selected sample concentration as per the formula below. Samples which fell within 80-120% of the measured neat concentration range were determined to display parallelism.

$$\text{RE (\%)} = \frac{\text{Measured analyte concentration} * \text{dilution factor}}{\text{Measured MRD concentration}} \times 100$$

3.4.3.2 Results

Parallelism of 4 endogenous samples (two high concentration, one medium and one low) were analysed at neat, with 2-fold dilution to a final 1/32 dilution. Back adjusted concentrations displayed linearity once samples were diluted to 1 in 8, suggesting matrix effects existed, inhibiting the accurate measurement of endogenous antigen in sample matrix. Using the 1 in 8 dilution as an anchor point, the relative error of subsequent dilutions were compared against this to demonstrate that samples then would fall within the 80-120% accepted range. Using this technique, all subsequent dilutions were within acceptable criteria demonstrating parallelism. The MRD was therefore set at 1 in 8 for further validation experiments.

Figure 3.13: Peripherin parallelism and MRD definition



A= parallelism experiment demonstrating adjusted concentration increasing to a dilution of 1 in 8, then reaching a plateau for further 2 dilutions. B= Using 1 in 8 dilution as the MRD, the relative error in subsequent dilutions were within the accepted 80-120% limitations.

3.4.4 Spike recovery

3.4.4.1 Methods

Three healthy control samples with known low levels of peripherin (<100 pg/ml) were selected for spike recovery. Each sample was diluted 1 in 8 (the defined MRD from above) with the selected assay sample diluent (lysate diluent B), and then divided into four aliquots. Three aliquots were spiked with peripherin recombinant across the linear range of the calibration curve at a high (20,000 pg/ml), medium (5000 pg/ml) and low (1000 pg/ml) concentration, ensuring spiked recombinant volume was under 10% of the total sample volume. The same volume of peripherin free diluent was added to the neat sample (fourth aliquot) to compensate for dilution. All samples were analysed in the same run. The % recovery of back calculated concentrations was determined by the two formulas below with an acceptable range of recovery at 80-120%.

Recovery 1

$$\text{Recovery (\%)} = \frac{\text{Analyte concentration measured in spiked sample- analyte concentration in neat sample}}{\text{Analyte concentration measured in diluent alone}} \text{ x100}$$

Recovery 2

$$\text{Recovery (\%)} = \frac{\text{Analyte concentration measured in spiked sample- analyte concentration in neat sample}}{\text{Expected concentration}} \text{ x100}$$

3.4.4.2 Results

Spiked blood samples were calculated by subtracting measured peripherin levels in the non-spiked blood sample from that of the spiked sample. Recovery was then calculated both against the measured level of peripherin in the diluent only spike (recovery 1) and also against the expected concentration (high=20,000 pg/ml, medium= 5000 pg/ml and low= 1000 pg/ml). Spike recovery experiments demonstrated very good recovery with values all within 80-120% of selected parameters.

Table 3.6: Results from spike recovery

Sample	Spike	Spike in blood* pg/ml)	Spike in diluent (pg/ml)	Recovery 1 (%)	Recovery 2 (%)
1	High	21420	19562	**109**	**107**
	Medium	6650	5821	**114**	**133**
	Low	1048	879	**119**	**105**
2	High	19868	21219	**94**	**106**
	Medium	4732	5045	**94**	**100**
	Low	1007	1145	**88**	**115**
3	High	23445	22123	**106**	**117**
	Medium	4675	4865	**96**	**94**
	Low	984	964	**102**	**98**

*High spike= 20,000pg/ml, medium spike = 5000 pg/ml, low spike =1000 pg/ml. Recovery 1 compared against spike in diluent, recovery 2 compared against expected concentration. *spike in blood measured as analyte of concentration measured in spiked sample- analyte concentration in neat sample.*

3.4.5 Dilution linearity

3.4.5.1 Method

Dilution linearity was performed to demonstrate that a sample with a high spiked concentration can be diluted and still provide a reliable result. This determines to which extent the dose-response of the analyte is linear in diluent within the range of the standard curve. [187] Three samples of healthy control blood were spiked with peripherin recombinant to a concentration of 100,000 pg/ml and diluted 1:2 with assay diluent to a final dilution of 1:32. All samples were diluted by the MRD and analysed in the same run. Back calculated concentrations were then compared to the expected concentration and % recovery calculated. A % recovery which fell within 80-120% of the expected concentration was deemed acceptable for this assay.

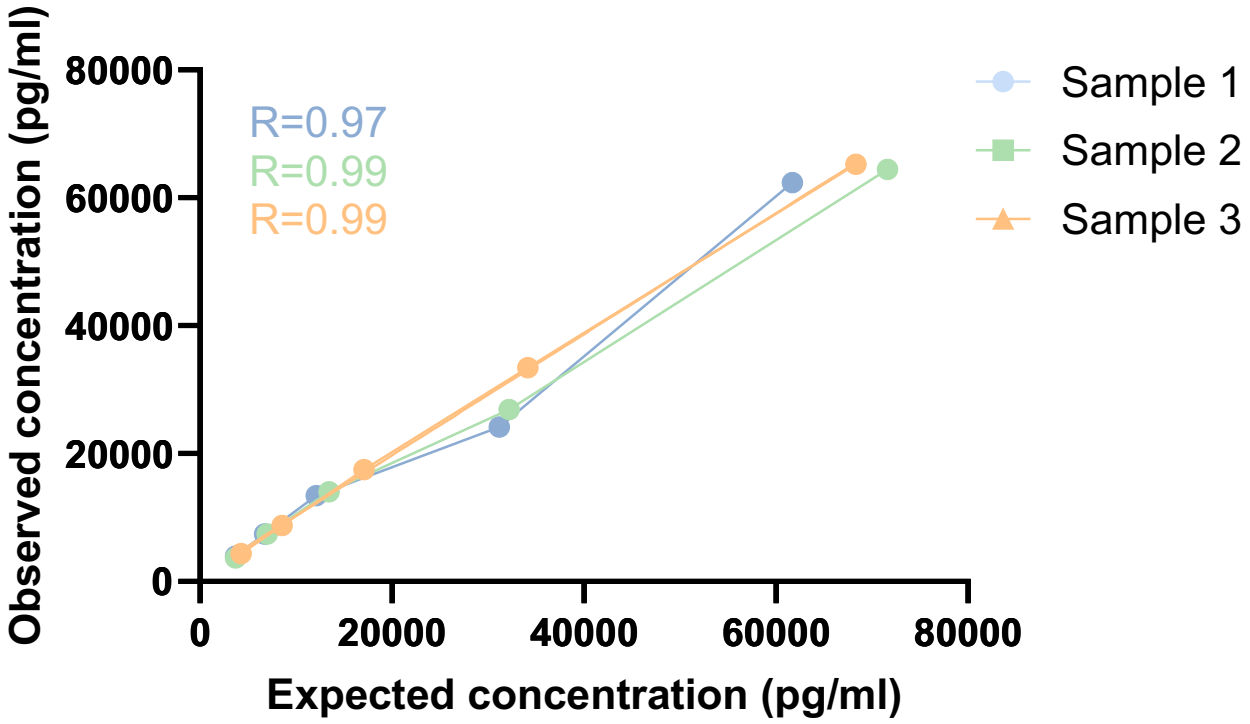
3.4.5.2 Results

Three samples were spiked with 100,000 pg/ml peripherin and diluted 1 in 2 five times, with the back calculated concentrations and % recovery displayed below in table. % recovery was within acceptable limits 12/18 times, with an overall average of 114%. Figure 3.14 displays the linear relationship between observed and expected concentration upon sample dilution, demonstrating linearity with $R^2 > 0.97$.

Table 3.7: Results from dilution linearity

Sample	Dilution factor	Observed conc (pg/ml) x DF	Expected conc of neat (pg/ml)	Recovery %
1	Neat	123445	-	-
	1:2	62345	61722.5	**101**
	1:4	24180	31172.5	**78**
	1:8	13461	12090	**111**
	1:16	7425	6730.5	**110**
	1:32	3951	3712.5	**106**
2	1:2	143223	-	-
	1:4	64443	71611.5	**90**
	1:8	26872	32221.5	**83**
	1:16	14033	13436	**104**
	1:32	7438	7016.5	**106**
	1:64	3722	3719	**100**
3	1:2	136644	-	
	1:4	65223	68322	**94**
	1:8	33455	34161	**97**
	1:16	17489	17080.5	**102**
	1:32	8764	8540.25	**102**
	1:64	4376	4270.125	**103**

Figure 3.14: Dilution linearity comparing expected and observed peripherin concentrations



3.5 Testing clinical samples

3.5.1 Introduction

At this early stage of assay development, testing clinical samples was used primarily to determine whether the assay performed to its expectations. Information gathered during this exercise should feed in to further assay optimisation in order to improve the assays' ability at detecting and measuring disease. The following experiments therefore aimed to demonstrate firstly that the assay had the ability to detect levels peripherin release in patients with peripheral nerve disease, compared to that of CNS disease and

healthy controls, and secondly that levels were higher in more severe cases of neuropathy. Neurofilament light was also tested for the same cohort of patients to determine whether peripherin correlates with neurofilament, but also whether it has the additional benefit of differentiating between PNS and CNS disease.

3.5.2 Methods

3.5.2.1 Study participants

A number of different pre-treatment patient samples were used in this clinical validation study as broad categories to help further determine the performance of the assay. The different disease groups and the sample repositories they came from are detailed below.

3.5.2.1.1 POEMS syndrome

Participants recruited to this study were selected from the UK POEMS database. Participants were adults (>18 years) who presented between 1999 and present day who fit the internationally recognised diagnostic criteria for POEMS syndrome including the two mandatory criteria, plus >1 major and >1 minor criteria (see table 1).[9] Participants in whom sera were stored at pre-treatment, post-treatment and relapsed disease were selected as a priority, followed by those with pre and post-treatment sera only. Some patients had multiple samples stored in the neuromuscular neuroimmunology biobank. The most recently collected sample prior to treatment initiation was deemed pre-treatment. Pre-treatment cases had no history of chemo or immunomodulatory therapy up to 12 months prior to samples being collected. Post-

treatment samples were taken between 6-12 months following therapy, in which the first sample collected in that time frame was selected. This time frame was selected as this is within a reasonable timeframe when patients clinically make improvements and other disease markers such as paraprotein levels respond. Overall neuropathy limitation scale scores were recorded for each case within 6 months prior to therapy, and at 3 years following treatment (if available, otherwise on most recent follow up).

Relapsed cases were defined by a biochemical relapse as a mandatory criterion with one or more of the below;

Mandatory criteria:

1. Biochemical relapse

    Persistent (≥2 recordings) serum VEGF elevation >771pg/ml from a previously normal result.

    If the lowest posttreatment value was >771pg/ml (i.e. not normalised), a persistent rise in serum VEGF of 50% from the lowest post treatment value was defined as relapse.

Plus one or more of:

2. Clinical relapse

   Re-emergence or worsening of clinical diagnostic features of POEMS syndrome or constitutional symptoms of weight loss, fatigue, sweats etc as defined by physician qualitative reports.

3. Haematologic relapse

   Re-emergence of serum/urine M-protein if undetectable or increase by 25% from lowest post-treatment sample.

   Increase in bone marrow plasma cell % on repeat bone marrow biopsy (if performed).

4. Radiological relapse

   Increase in the size or avidity of lesion or new plasmacytomas

3.5.2.1.2 Acute inflammatory demyelinating neuropathy (AIDP)

Cases of AIDP (GBS) from 2015 to present day were collected as part of a study approved by the ethics committee at the Medical University of Vienna (EK1283/2018). Patients meeting the level 1 or 2 Brighton diagnostic criteria for GBS were included, aged over 18 with no other pre-existing or current neurological diagnoses.

3.5.2.1.3 Acute motor axonal neuropathy

Cases of acute motor axonal neuropathy (AMAN) and acute motor sensory axonal neuropathy (AMSAN) were provided from a biobank of Guillain-Barré syndrome cases (and variants) recruited from the International GBS outcome study (IGOS). Cases of AMAN and AMSAN were defined neurophysiologically. Samples were from patients over 18, however no further criteria were set for inclusion. GBS disability scores were also provided for each case.

3.5.2.1.4 Central nervous system disorders

Peripherin is present in the spinal cord (figure 3.7), possibly contained within the proximal nerve roots which enter the cord rather than strictly being part of the CNS, or within the anterior horn cell bodies. For this reason, cases with dementia (Alzheimers, frontotemporal dementia, semantic dementia and logopenic progressive aphasia) were selected as optimal cases for the CNS disease group, as dementia is not thought to damage the spinal cord (which may result in peripherin release), compared to other CNS disorders such as multiple sclerosis, for example.

A cohort of samples were provided by the University College London Wolfson CSF prospective cohort (ethics code 12/0344), with a preference towards cases aged between 40-70 years old to match that of the POEMS cohort of mixed dementia types.

3.5.2.2 Sample collection and processing

Due to samples being donated from different biobanks, standardised sample collection and processing was not achievable. However, all samples were serum, centrifuged within 6 hours of collection, and frozen to <-20°C. No sample had been freeze-thawed over one previous occasion.

3.5.2.3 SiMOA immunoassay

The SiMOA immunoassay steps were carried out according to the Quanterix Sample Preparation User Guide. [146] In short, bead reagent solution was prepared by mixing helper beads (Quanterix) and 0.3 mg/ml antibody (MC6, Sigma) conjugated capture beads in a proportion of 150K to 350K. Beads were washed two times and then resuspended in bead diluent (Quanterix). Detector antibody was prepared by biotinylating the monoclonal antibody MC3 in 60-fold-molar excess of EZ-Link NHS-PEG4-Biotin (Thermofisher), and diluted to a concentration of 1 ug/ml for the assay. SBG was then diluted using SBG diluent (Quanterix) to a concentration of 50pM. Beads, detector, SBG and RGP were loaded into the SiMOA HD1, using the homebrew 2-step assay parameters previously defined.

Peripherin recombinant #3 was diluted with assay diluent (lysate diluent B) to a top concentration of 250,000 pg/ml, diluted 1:5 to 80 pg/ml at the lowest calibrator and a blank. Samples were thawed in room temperature for an hour, centrifuged at 14,000 g for 5 minutes and 35 ul aspirated from the centre of the collection tube, diluted with 242 ul of lysate diluent B (for a 1 in 8 dilution) and vortexed for five seconds. 280 ul of

calibrator or sample was added to a Nunc™ 96-Well Polypropylene Storage Microplate (Thermo Scientific) and run in duplicate.

3.5.2.4 Neurofilament immunoassay

Quantification of neurofilament light (NfL) was performed using the SiMOA NF-light commercial assay kit (Quanterix) run on the SIMOA HD-1 analyser according to manufacturer's instructions. This assay kit has been validated to be highly reproducible, with within run CVs of approximately 7-9% and between runs of 3.8-4.1%.[188] For this reason, samples were run as singlets to allow for a greater number of samples to be tested overall. Two control samples were included to ensure assay precision.

3.5.2.5 Statistical analysis

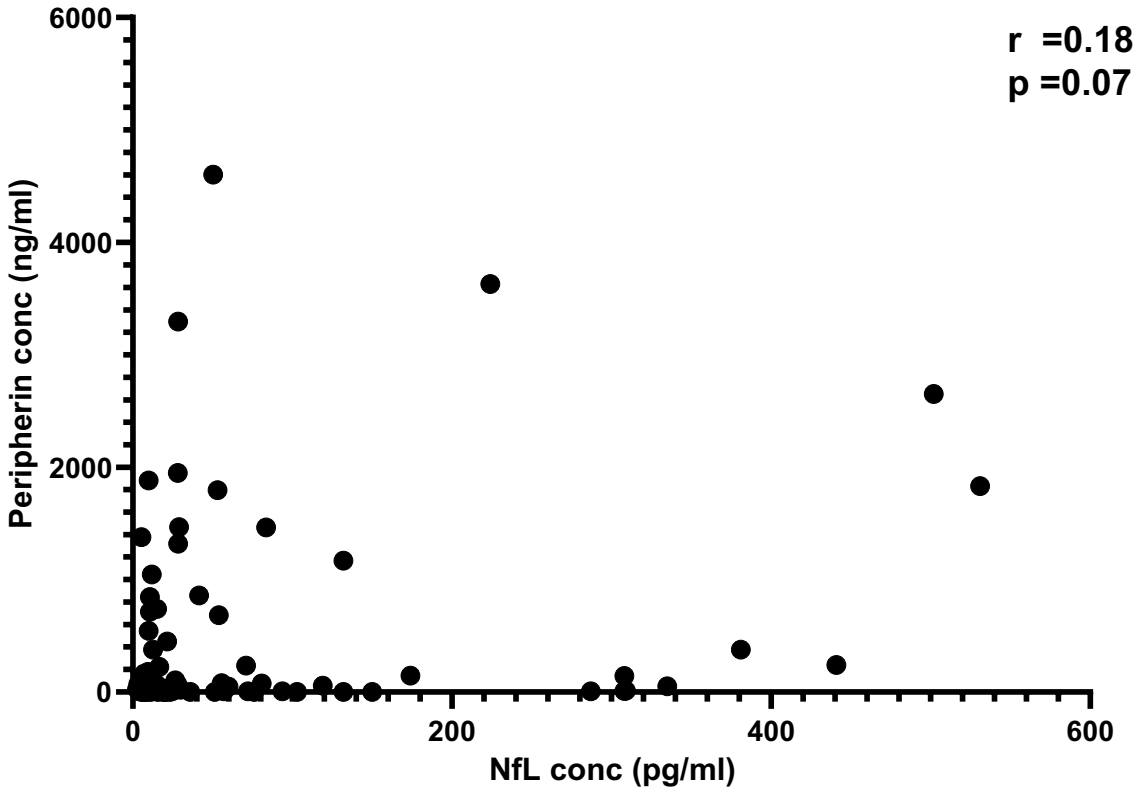
Because peripherin levels were not normally distributed (Shapiro-Wilk test, $P < 0.05$), non-parametric statistics were used for analysis. Kruskal-Wallis tests were used to identify whether statistically significant differences in peripherin levels existed between groups. Statistically significant results ($P < 0.05$) were followed by Mann-Whitney U tests to investigate individual group differences. Simple linear regression was utilised to determine whether a relationship existed between levels of biomarkers, and from each biomarker to disease severity scores (ONLS, GBS disability score).

3.5.3 Results

Thirty-five matched pre- and post-treatment POEMS samples were tested for peripherin, as were 15 matched relapsed cases. 15 AMAN/AMSAN cases, 10 AIDP, 30 CNS and 20 healthy controls were also tested.

Firstly, levels of peripherin were compared to levels of NFL recorded for the same samples to determine whether peripherin levels correlate with NfL in peripheral nerve disease. All POEMS syndrome, AMAN/AMSAN, AIDP and healthy control samples in which both NfL and peripherin were available (n=95) were plotted (see figure 3.15). A Spearman's correlation coefficient was calculated which demonstrated the two biomarkers did not correlate significantly (r=0.18, p=0.07).

Figure 3.15: Correlation between peripherin and NfL levels

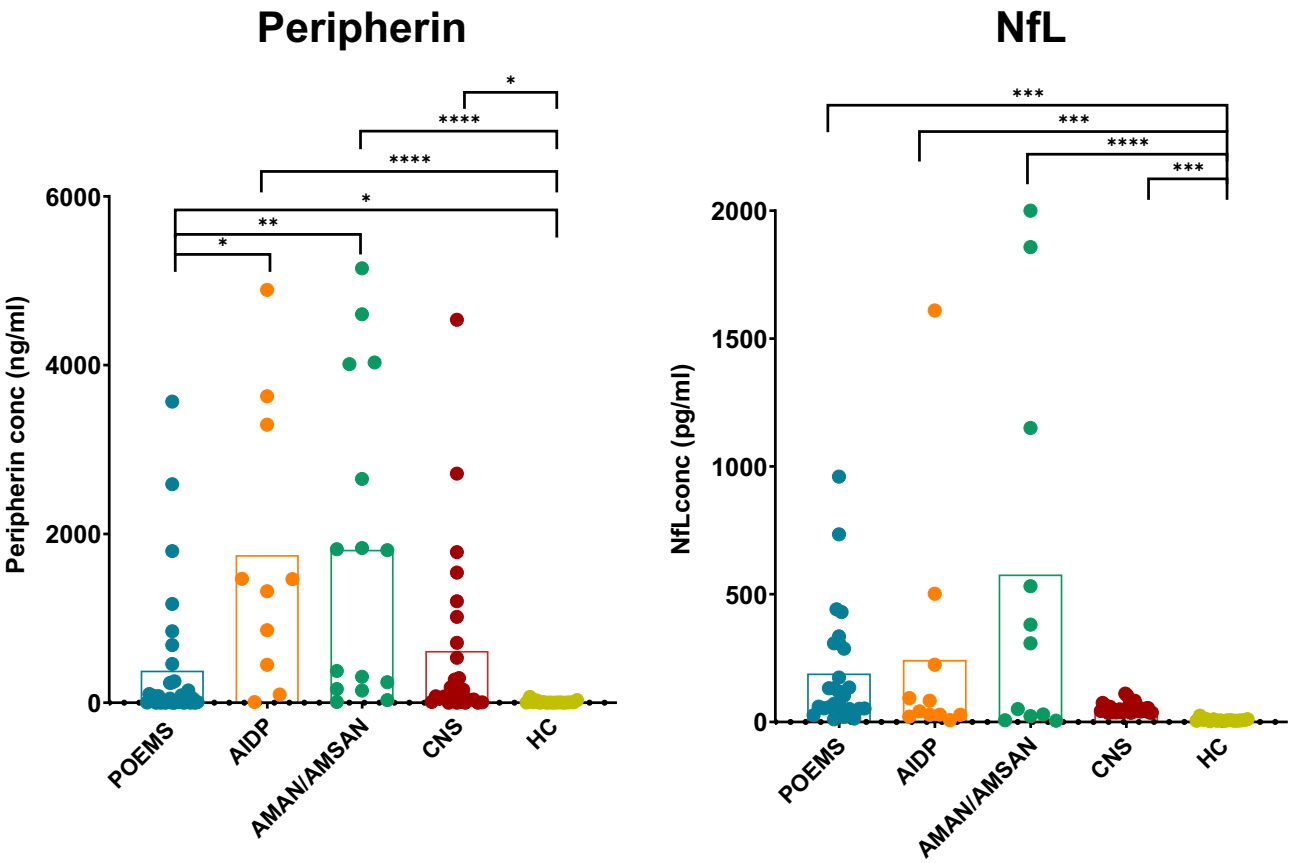


Concentration of samples in which both peripherin and NfL were tested simultaneously for correlation. Note 5 points were not included in the graph as outside the axis limits.

Figure 3.16 compares levels of peripherin and NfL in pre-treated POEMS cases compared to that in the other pre-treatment disease cohorts. Firstly, levels of peripherin were recorded at far higher concentrations than had been initially suspected from prior studies. Levels recorded were ranging from 144 pg/ml to 5146355 pg/ml) and hence data are displayed as ng/ml for clearer presentation. Reassuringly, levels of peripherin in healthy controls were the lowest of the cohort (10.5 ng/ml, 95% CI 0.8-20.2) indicating the lack of peripheral nerve damage in this group of patients. Levels of

peripherin were significantly raised in POEMS syndrome (380.2 ng/ml, 95% CI 93.8-666.7) compared to that of healthy controls ($p<0.023$) indicating the assay was able to detect a degree of peripheral nerve axonal loss in this cohort of patients. All forms of Guillain-Barré Syndrome (AIDP, AMAN/AMSAN) had significantly raised levels of peripherin compared to all other groups. GBS is a severe, acute onset neuropathy often with significant axonal loss and disability, and hence levels being more raised in such cases (AIDP= 1748 ng/ml, 95%CI 571-2926), AMAN/AMSAN= 1812 ng/ml, 95% CI 785-2838) would be expected. Axonal forms of GBS (AMAN/AMSAN) were marginally higher than those with AIDP which is acute but predominantly demyelinating at onset, but this finding did not reach statistical significance. Levels of peripherin in patients with CNS disease were higher than that of healthy controls (612 ng/ml, 95% CI 169-1055 versus healthy controls of 10.54 ng/ml, 95% CI 0.8-20.2) which was an unexpected finding, since peripherin is thought to be mostly released by peripheral rather than central neurones. The mean peripherin level in CNS disease cases was higher than that of the POEMS cohort (612 ng/ml, 95%CI 169-1055 in CNS disease versus 380.2 ng/ml, 95% CI 93.8-666.7 in POEMS syndrome), however this was not significant ($p=0.18$). These data were compared to the same serum samples tested for NfL. Firstly, levels of NfL in all conditions were significantly greater than in healthy controls. Levels of NfL were greatest in the AMAN/AMSAN cohorts (575 pg/ml, 95%CI 71.9-10801) which present with acute axonal peripheral nerve damage. This was followed by AIDP (242 pg/ml, 95% CI -77-562). POEMS syndrome displayed increased levels of NfL (189 pg/ml, 95%CI 102-277) compared to healthy controls (7.9 pg/ml) also, but less so than the more acute forms of neuropathy which often result in worse outcomes. CNS patients also demonstrated raised levels of NfL (52.4 pg/ml, 95% CI 45.3-59.5) compared to healthy controls, but at lower levels than the PNS diseases as expected.

Figure 3.16: Levels of peripherin in diseases of the peripheral and central nervous system compared to healthy controls

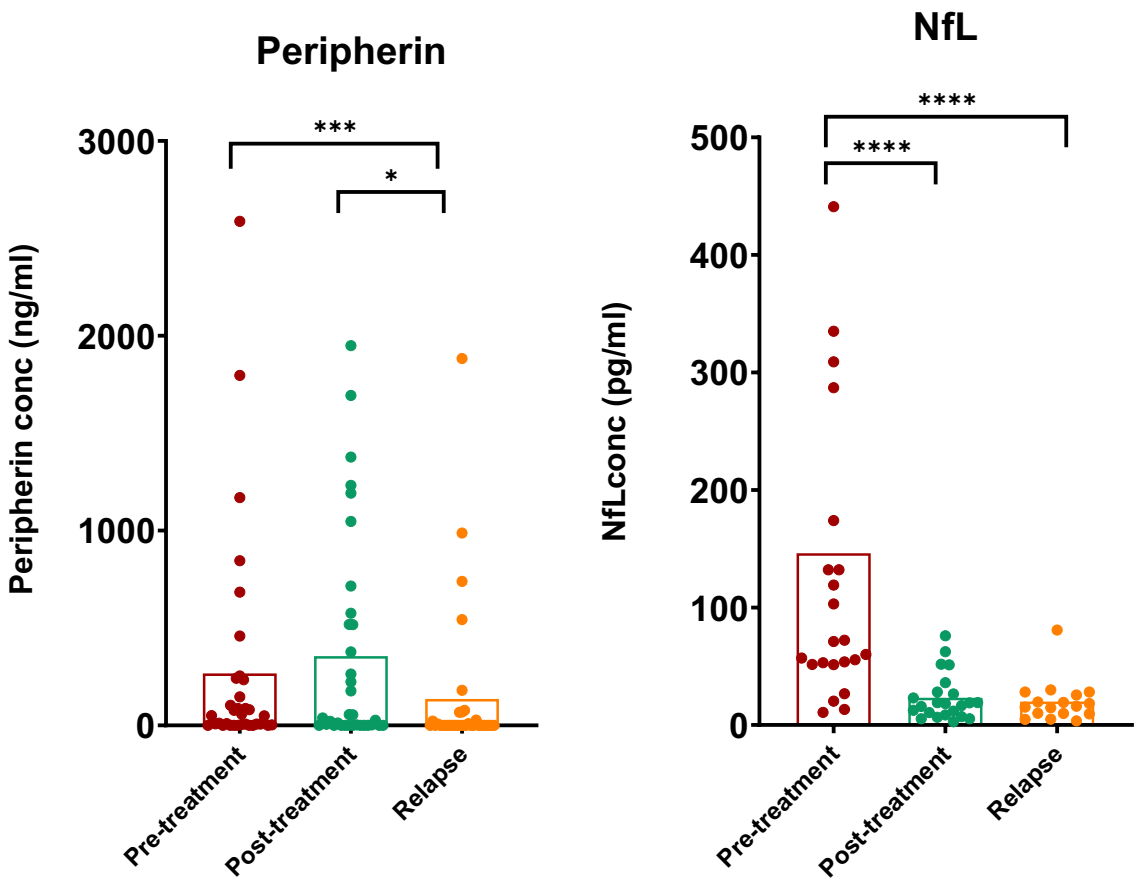


Levels of peripherin (in ng/ml) and NfL (pg/ml) in POEMS syndrome, acute inflammatory demyelinating neuropathy (AIDP), acute motor axonal neuropathy (AMAN) and acute motor sensory and motor neuropathy (AMSAN), central nervous system disease (CNS) and healthy controls (HCs). Bars depicting the mean, dots representing individual samples.

Next, peripherin and NfL were measured in POEMS cases pre-treatment, post-treatment and at relapse to determine whether levels reflected underlying pathological changes to the nerves. The details of this are shown in figure 3.17. Levels of peripherin were not shown to change significantly from pre- to post-treatment. Levels did however significantly reduce from pre-treatment (266 ng/ml, 95% CI 69.6-463.8) and post-

treatment (355 ng/ml, 95% CI 163.4-547.5) to relapse (135 ng/ml, 95% CI 2.81–269.6) which was unexpected and does not reflect the expected disease activity. Comparing this to NfL, levels were markedly raised in the pre-treatment cohort (146 pg/ml, 95% CI 71.6-220.8) compared to that of post-treatment (23.1 pg/ml, 95% CI 14.6-31.6), but these did not subsequently rise upon relapse either (19.9 pg/ml, 95% CI 10.7-29.0).

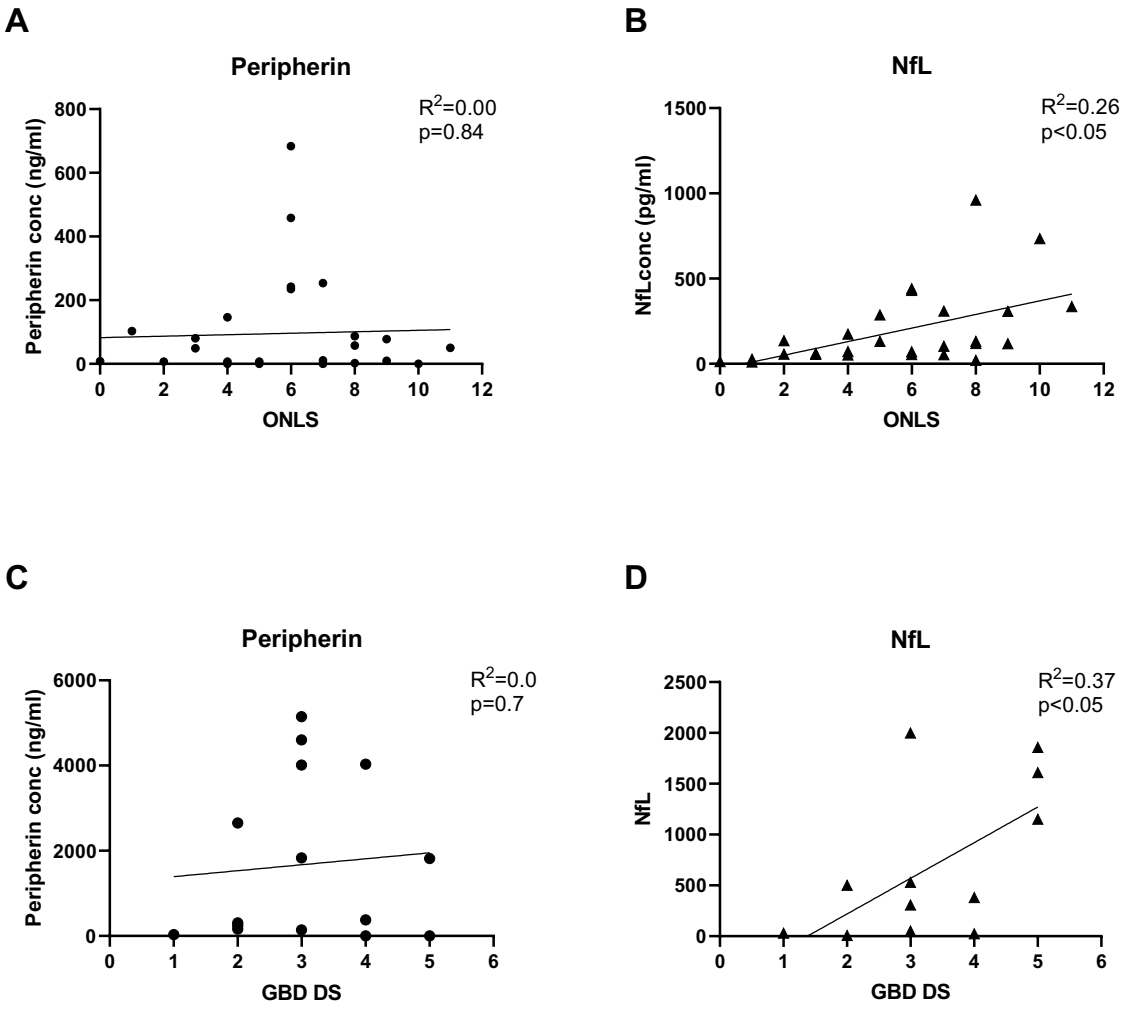
Figure 3.17: Levels of peripherin and neurofilament in pre-treated POEMS syndrome compared to post-treatment and relapse.



Levels of peripherin (in ng/ml) and NfL(pg/ml) in POEMS syndrome pre-treatment, post-treatment and upon relapse. Bars depicting the mean, dots representing individual samples.

Levels of biomarkers were also investigated to determine whether they could be used as potential measures of peripheral nerve damage by comparing against validated functional assessment tools. For this purpose, simple logistic regression was used to determine the relationship between functional outcome scores in both POEMS syndrome (the ONLS) and in GBS (the GBS disability scale) with levels of biomarkers measured. In both POEMS and GBS (using respective scores), increases in functional outcome scores (relating to increased disease activity/ disability) resulted in significant increases in neurofilament ($p<0.05$), which was not demonstrated in peripherin- see figure 3.18 for details. The interpretation of this discrepancy is detailed in the discussion section 3.6.5. In addition, levels of neurofilament light at presentation correlated with post-treatment ONLS level at 3 years for patients with POEMS syndrome ($R^2=0.24$, $p=0.008$), meaning that patients with greater axonal damage on presentation were less likely to recover long-term.

Figure 3.18: Levels of peripherin and neurofilament in response to increasing functional disability scales



Levels of peripherin and neurofilament related to increasing ONLS functional outcome score related to POEMS syndrome (A and B), and to GBS disability scores (GBS DS) in GBS (C and D).

3.6 Discussion

This chapter details a rigorous approach to developing a novel biomarker of axonal peripheral nerve disease from conception to clinical validation. Although the clinical data has shown that the assay is not yet performing to its required parameters, several important lessons have been learnt in the process. The following discussion highlights the difficulties encountered through homebrew assay creation, interprets the data gathered and establishes clear plans for future development.

3.6.1 Pre-analytical experimentation

There are no published data regarding the detection or measurement of peripherin in biological samples, and thus significant preanalytical experimentation was required to select the optimum antibodies and recombinant protein combination before transferring to solid phase platforms. These experiments were performed to determine whether peripherin antibodies were able to bind to and detect peripherin both as a recombinant protein, and in its endogenous folded protein form. This was of relevance for immunoassay development, because accurate measurement of unknown concentrations of endogenous analyte in biological samples rely upon the production of a calibration curve using known amounts of purified antigen to compare against. Although recombinant protein production is a mature technology, the process itself remains complex and is not standardised. Gene amplification, vector expression, transformation into host, isolation and purification processes differ and may result in proteins which may not accurately represent that of the endogenous form. Bacterial expression of proteins for example results in few to no post-translational modifications

(such as phosphorylation, acetylation or methylation). Such post-translational modifications that occur to endogenous proteins may result in specific tertiary or quaternary folding structures, which may result in differently exposed epitopes for antibody recognition compared to recombinants. Often, fragments of full-length proteins are provided as recombinants, which limits the antibodies that can be included to only those which bind to epitopes contained within that fragment.

The process of antibody production is also similarly intricate. Monoclonal antibodies are made by injecting animals (often mice) with an antigen (which can be a recombinant protein or epitope of the protein) to provoke an immune response, leading to antibody producing B cell production from the spleen. These cells are harvested and fused with immortal B (myeloma) cells to produce a hybrid cell line (hybridoma). These cells are then separated, screened, and those which produce the desired antibodies are cloned. Such clones can then be grown indefinitely in culture medium or can be injected into the peritoneal cavity of mice which allows cells to grow and release antibodies into ascitic fluid. Antibodies are then separated and purified by ion exchange chromatography, size exclusion chromatography, protein A/G affinity chromatography or affinity purification. Protein stabilisers are then added to antibodies such as sodium azide, bovine serum albumin (BSA) or glycerol, for example. Resulting antibodies vary in terms of species reactivity, isotype, binding affinity, recommended application, purity and preparation, some of which are not appropriate for particular downstream applications. For example, BSA buffer is often added to antibodies as a protein stabiliser for long term storage. However, BSA competes with antibodies and greatly reduces bead conjugation efficiency and biotinylation, steps fundamental to the process of signal amplification in

SiMOA technology. In addition, the process of bead conjugation and biotinylation requires large amounts of antibody (around 150 micrograms) for each step, which again limits which antibodies can be used based upon yield and respective cost (for example, some antibodies only contain 20ug antibody per vial). Ideally, antibodies selected for SiMOA need to be monoclonal (polyclonals often have lower affinity and display lot-to-lot variability) at relatively high concentration, demonstrate no cross-reactivity with non-target analytes, display high affinity, are affinity purified and carrier free, ideally in PBS. It is unlikely that any commercial antibody has all the above characteristics, and thus the best available are selected. An alternative method to the above would be to produce custom antibodies, whereby the selected recombinant could be used as the immunogen for antibodies to be made against, and produced to specification, however this process is clearly more time consuming and costly.

The initial western blot, dot blot and immunoprecipitation experiments were sufficient to demonstrate that antibodies to peripherin bind to recombinant protein and peripheral nerve endogenous proteins. These initial experiments were performed because ELISA based techniques were initially not successful, and therefore further antibody and recombinant protein analysis (whilst removing potential confounders of solid phase assays) were necessary endeavours. Also, proving that peripherin antibodies did not bind to central nervous system structures (and that neurofilament-light antibodies do) was also a particularly important pre-analytical step.

3.6.2 Transferring to solid phase

Transferring the antibody and recombinant protein combination to an ELISA platform poses several challenges. Utilisation of a solid phase assay plate introduces factors which may influence antibody-antigen interactions and resulting signal production. For example, when setting up a sandwich ELISA, the passive binding of capture antibody to the ELISA plate is influenced predominantly by the molecular interactions of the antibody with the polymer surface structure of the plate. Plate surfaces vary in the degree of hydrophobicity, in that increasing hydrophilicity increases electrostatic interactions which can play a significant role influencing which biomolecules bind strongly to the surface. Generally, hydrophobic plates bind lipid rich molecules compared to hydrophilic which bind immunoglobulins and other hydrophilic proteins.[189] Selecting the incorrect plate will result in no capture antibody binding, and thus the subsequent sandwich ELISA fails. However, this is only one of several potential interactions; capture antibody diluent, incubation times, choice of block, wash composition and technique, antibody and recombinant diluent, antibody combination, concentration, efficacy and sensitivity and specificity of secondary HRP conjugated antibody are all considerations which have to be carefully controlled for and then varied in turn as the independent variable to assess the effect on signal and noise production. Selecting the best combination of antibodies appears to be the most significant factor determining the success of the immunoassay.

3.6.3 Developing the assay for SiMOA technology

When setting up a SiMOA immunoassay, as mentioned before, monoclonal antibodies are preferable. For this reason, when using ELISA to select the optimal antibodies to take forward, each monoclonal (mouse) antibody was tested in turn as a capture antibody whilst using the same polyclonal (rabbit) antibody to determine which monoclonal resulted in the best signal:noise ratio (as all other variables were controlled for). Ideally, testing each monoclonal antibody as capture and then also as detector in a checkerboard combination would be the most rigorous method to determine the optimal monoclonal antibody combination to take forward for SiMOA.[177] Same species capture and detector can be used in SiMOA technology since the assay utilises biotinylated antibodies which are then bound to by streptavidin-β-galactosidase enzyme. However, since all the monoclonal antibodies available were mouse origin, the detectors would have to be biotinylated or HRP-conjugated prior to use in ELISA in order to prevent anti-mouse HRP conjugated antibody binding to the mouse capture and creating falsely positive signal. Such processes are technically feasible, however, they were felt to be too costly and time consuming, and may in fact introduce further variability, so were discounted. Regardless, MC6 and MC3 appeared to create the most signal when tested independently as captures, which was also the case using the SiMOA with MC6 capture (pre-bound to beads) and MC3 (biotinylated) detector.

Once the best combination of antibodies had been determined, and assay parameters optimised, the SiMOA immunoassay displayed a calibration curve which spanned a broad dynamic range with a limit of detection at 11.3 pg/ml. This was a significant improvement compared to the ELISA of 332 pg/ml. SiMOA technology can increase

sensitivity of assays by up to 1000x than that of ELISA, so this improvement was significant but perhaps could still be improved further. Regardless, this sensitivity was deemed acceptable for ongoing development based upon comparisons with neurofilament light serum levels which are often in the region of 20 pg/ml in healthy controls.[190]

3.6.4 SiMOA assay validation

When testing spike recovery and parallelism, which essentially determines whether matrix effects exist between the calibration curve and clinical samples, the assay did not perform to acceptable standards. For this reason, a range of assay diluents were required to be tested, at a range of dilutions in order to mitigate such effects. In doing so, the assay sensitivity decreased, with a LOD of 120 pg/ml and LLOQ of 339 pg/ml. This was likely due to a combination of a flattening of the calibration curve, and also due to increased background signal produced by using a diluent which had higher concentrations of protein (although the exact composition was commercially protected). Such alterations to the diluent were successful in that they improved matrix effects, demonstrating spike recovery, parallelism and dilution linearity within acceptable parameters.

Another striking and unexpected finding on testing clinical samples during the validation steps (once using lysate diluent B) was that levels of peripherin were recorded far higher than expected in endogenous samples at the ng/ml range, and even when using healthy controls (around 1000-5000 pg/ml). Despite not knowing the true

concentration of peripherin in human samples, this value was far higher than predicted, which requires further investigation into why this may be. Validation experiments using lysate diluent B were producing data which passed the criteria for acceptance for example in parallelism and spike recovery, as these validation steps measure specific features of assay dynamics rather than identify the accuracy of the reading itself. For example, the recorded concentration may be demonstrated to be 10x the actual concentration of the sample, but as long as the concentration reduces by half upon 1:2 dilutions the assay will pass the criteria for parallelism. High signal readings may be due to non-specific binding of the immunoassay antibodies to non-target proteins in the sample matrix (sample and diluent) leading to false positive signal production. However, Western blot experiments suggested that monoclonals to peripherin were both sensitive and specific, and therefore this finding was surprising. In addition, background AEB values of blanks using lysate diluent B were not dissimilar to other diluents which would suggest there is little antibody binding to diluent constituents. Although this assay diluent appeared to perform well in the validation experiments, such experiments are not absolutely essential to immunoassay development. Some assays for example may demonstrate poor parallelism, but as long as all samples are diluted by the same factor and compared, meaningful results can still be gleaned. More work is required to further investigate why such high levels of peripherin were recorded, whether non-specific binding exists and whether diluent composition could be changed to improve such results. For example, two alternative diluents recorded levels of peripherin in endogenous samples much lower to that than the finally selected diluent (lysate diluent B). For the same endogenous sample, the level of peripherin recorded in lysate diluent B was 27,081pg/ml compared to diluent A which was 2247 pg/ml and diluent E 1509 pg/ml. Without knowing the true concentration of peripherin

in the endogenous sample means it is very difficult to know which diluent is recording the levels most accurately. Going forward, diluent A and diluent E can be further explored to see whether sample measurements are thought to be more accurate and reliable, and display similar characteristics on validation experiments such as parallelism. Being able to detect the true (or close to true) value of peripherin in an endogenous sample by other means (such as ELISA) would be most useful to select the most appropriate diluent to then optimise.

3.6.5 Testing clinical samples

3.6.5.1 Neurofilament light

To interpret the results of the peripherin clinical samples, analysis of neurofilament light (NfL) levels was performed primarily as the validated 'gold standard' biomarker of axonal pathology.[123,131,132,191] Firstly, levels of NfL were significantly raised in patients with pre-treatment POEMS syndrome compared to healthy controls (figure 3.16), and correlated with neurological disease severity as measured by the ONLS score (Figure 3.18). This finding has not been reported elsewhere in the literature, although was expected from our knowledge of peripheral nerve pathogenesis and neurophysiological data. This study has also demonstrated that high levels of pre-treatment/ presenting NfL correlate with a significantly worse neurological outcomes as defined by ONLS ($p=0.008$), and thus NfL may be considered both a marker of underlying peripheral nerve damage and a predictor of poor outcome in POEMS syndrome. Comparing NfL levels to other disease cohorts, NfL levels were similar to that of AIDP which although is a more acute and often aggressive disease, is characterised by more demyelination than early axonal loss. Serum NfL levels in CNS cases were significantly lower than that of

PNS disorders, due to the lack of CNS derived NfL reaching the systemic circulation, however levels remained significantly greater than that compared to healthy controls (52.4 pg/ml compared to 7.9 pg/ml). Interpreting such findings, although a peripheral nerve specific biomarker would be of considerable benefit (as detailed in the introduction), when testing serum NfL, it is perhaps unlikely that CNS derived NfL would significantly confound the results from patients with peripheral nerve diseases. It may in fact be more useful when examining CNS disorders, in which peripheral nerve derived serum NfL would certainly confound results.

Studying NfL in treated and relapsed disease, it is clear to see that treatment significantly halts further axonal damage, in which levels of NfL are reduced to levels similar to that of healthy controls (23.2 pg/ml compared to 7.9 pg/ml). In clinical practice this may be of particular use. Following POEMS directed therapies, neurophysiological findings do not typically reflect active axonal disease, and will be characterised by the degree of existing neuronal damage. For this reason, neurophysiology is not useful for many months (if not years) following treatment, which may then demonstrate a degree of improvement, or at least stability compared to previously deteriorating neurophysiological studies. However, this study, in which patients' post-treatment serum sample was taken between 6 and 12 months post treatment, has demonstrated a normalisation of NfL far before any meaningful change would be detected on neurophysiology. It may therefore be of use to measure levels of NfL clinically post treatment to ensure levels have dropped substantially, whereby failed suppression might indicate treatment resistance or failure. When looking at relapsed disease, levels of peripherin do not significantly increase from post-treatment

levels (19.9 pg/ml in relapse compared to 23.2 pg/ml following therapy). This is likely due to the fact that following treatment, patients are closely followed up with serial VEGF, SPE, IF and SFLC analysis, and monitored closely for recurrence of clinical features such as oedema, papilloedema, neuropathy and skin lesions. Anecdotally, most cases of treatment resistance or relapse are first identified by biochemical analysis, and thus at this stage it is unlikely neuropathy has started developing. The high levels of NfL (and development of neuropathy) in pre-treated disease is likely therefore a manifestation of a long standing undetected haematological dyscrasia, raised pathogenic cytokines and progressive and severe damage to peripheral nerve structures.

3.6.5.2 Peripherin

Overall, levels of peripherin as measured by the novel homebrew SiMOA immunoassay do not correlate with that of NfL, indicating the assay is not accurately measuring peripheral nerve disease in the same way that NfL does. Although this may be the case, levels of peripherin measured in neuropathy are higher than that of healthy controls, and appear to reflect disease severity in a similar fashion to NfL, with higher concentrations in GBS compared to POEMS syndrome. Although levels of such may be inaccurate, relative comparisons between groups does indicate some degree of the assay functioning. However, peripherin levels do not appear to reduce following therapy, and do not correlate with neurological disease severity in either POEMS syndrome or GBS as NFL levels do, indicating the assay is not functioning to its prespecified requirements. A clearer indication of the peripherin assay not functioning to its expected specification is the level of peripherin measured in cases with CNS

disease, which appears to be of similar magnitude to that of peripheral nerve diseases with very high levels recorded (in the ng/ml range (612 ng/ml)). Previous published reports and data from the initial Western Blot experiments would support the fact that little to zero peripherin is identifiable in most CNS structures by this technique compared to peripherin identifiable from peripheral nerve structures. Therefore the raised levels in the SiMOA assay remain unexplained. Although conceivably there may be some peripherin released from CNS structures, it would be expected that regardless of this, comparatively far greater levels would be released in peripheral nerve diseases, such as it is for NFL. Considering reasons for false positive results, diluent choice has been discussed above and is likely of most relevance. Another potential mechanism may be that the assay antibody pair is binding to and detecting non-peripherin structures in the CNS samples. It may be that such structures are potentially released from the CNS during damage. The first consideration would be whether the antibody combination is falsely detecting NfL protein as a similar neurofilament protein. To investigate this further, peripherin levels were measured using NfL, GFAP, UCH-L1 and Tau low and high concentration calibrator (n=4) and control samples (n=4) from a neuro 4-PLEX B assay kit (Quanterix) of biomarkers of traumatic brain injury (not shown in results). Levels of peripherin were undetectable in every case, demonstrating the assay is not falsely detecting other neuronal damage biomarkers. It may be however that the peripherin antibodies remain to bind to an unknown CNS derived neuronal protein. However, as mentioned before, antibody specificity appeared high on western blotting (figure 3.7) with no additional bands which refutes this hypothesis.

In summary, the SiMOA homebrew peripherin immunoassay appears to measure recombinant peripherin protein to a high level of sensitivity with a broad dynamic range. However, clinical testing of samples has lacked correlation with NfL, the considered 'gold standard', neuropathy clinical outcome measures, and was unexpectedly raised in patients with CNS disease. Although the immunoassay appears to require further refinement, knowledge of peripherin function, release upon damage and dynamics within serum would be useful during further assay development.

3.6.6 Future assay development and optimisation

Future development of this assay will first require further experimentation to verify the capture and detector antibody specificity/selectivity. This can be performed by pull down depletion experiments, where an endogenous sample is tested after pre-mixing with an excess of assay capture beads/antibody, then removing the beads. This 'depleted' sample is then tested for peripherin and compared to the same 'non-depleted' sample. This should demonstrate that levels of peripherin significantly drop in the depleted sample, demonstrating the capture antibody binds specifically to the peripherin. Although this test in isolation does not completely prove sensitivity/ specificity, because if the capture antibody binds to a non-peripherin structure, it would likely pull this down, resulting in the sample appearing depleted also upon further testing. However if comparing antibodies, one which depletes a sample more efficiently would be advantageous to use as it would also suggest the antibody is in excess to antigen and efficiently binds. A second option would be to perform the same experiment but then to western blot the pull-down material to determine the constituents of the pull-down using Coomassie blue staining, and using peripherin antibody to confirm

successful pull down. It may also be worth considering collaborating with a laboratory that has experience in manufacturing antibodies, which can be produced to recommended specification (as described above) for further testing, which are expected to perform better than commercially available antibodies.

Determining the best antibody or diluent combination is more difficult to achieve without control samples in which levels of endogenous analyte are known, however spike recovery is useful (although may not accurately represent endogenous sample measurements), and a reasonable comparison can be made between control samples (which should be very low concentration) vs disease (which should be measurably higher but probably within the pg/ml range). Testing alternative diluents will be of considerable use. Firstly, testing a number of samples in which predicted levels of peripherin are low, medium and high with two or three diluents will be useful to delineate which diluent appears to measure levels most accurately (to predicted levels of peripherin). Once determined, testing how this diluent performs in validation tests such as spike recovery, parallelism, dilutional linearity would be necessary. It is also possible to make up and modify diluent composition using a combination of varying concentration/ molarity phosphate or tris, sodium chloride, tween and BSA, with the addition of a heterophilic blocker if the diluent performs poorly at validation (but otherwise appears accurate in measuring peripherin).

Developing highly sensitive immunoassays from conception typically requires teams of experienced laboratory staff with a large amount of funding and time. Although this immunoassay has not yet been developed to acceptable accreditation standards, the preliminary data and experience gained should enable further investment for further assay development to hopefully one day become a clinically relevant test. This study has successfully demonstrated through serum neurofilament-light measurement that axonal loss is a key feature of peripheral nerve pathology in POEMS syndrome. Serum NfL is additionally a sensitive biomarker of POEMS syndrome, which reflects disease activity, signifies successful disease suppression, and is a predictor of longstanding neurological disability (at three years post treatment).

4. Defining the cytokine networks of POEMS syndrome

4.1 Introduction

Although the pathogenesis of POEMS syndrome is yet to be determined, activation of a systemic hyperinflammatory cytokine network is postulated to result in downstream multi-system organ damage . In 1996, the discovery that vascular endothelial growth factor (VEGF), a vasopermeability cytokine, was found to be significantly raised in POEMS syndrome patients supported this hypothesis, and appeared to explain several of the associated vascular leak characteristics.[10] Following this discovery, as discussed in previous chapters, several studies have demonstrated the utility of VEGF as a biomarker of disease in POEMS syndrome diagnosis and monitoring, prognosis and response to treatment.[2,20,44,79,109,192]

Cytokines are small, cellular secreted proteins of ~5-20 kDa that have a specific effect on the interactions and communications between cells. They are produced by several cell types, including immune cells (macrophages, B and T lymphocytes and mast cells), endothelial cells, fibroblasts and various stromal cells.[193] Not all of the characteristic features of POEMS syndrome can be explained by endothelial hyperpermeability, and it is conceivable that several other cytokine networks are involved with their own distinct pathophysiological consequences. A number of studies have documented individual or small clusters of cytokines which are raised in POEMS syndrome patients, but little has been done to explain the underlying disease pathology, particularly that of neuropathy.

This chapter will begin by summarising the cytokines already considered to be potentially implicated in POEMS syndrome and their proposed pathogenic relevance. It will then detail a number of experiments designed to further explore the cytokine network of POEMS syndrome within a larger and more comprehensively characterised POEMS cohort, to improve understanding of the immune mediated processes which lead to disease.

4.1.1 Vascular endothelial growth factor

4.1.1.1 Physiological function of VEGF

Vascular Endothelial Growth Factor (VEGF) is a glycoprotein thought to be the key cytokine implicated in POEMS pathogenesis. Markedly elevated levels of VEGF are seen in POEMS syndrome, correlating broadly with disease activity and overall survival. [11,13]

There are five different types of VEGF; VEGF-A, placental growth factor (PLGF), VEGF-B, VEGF-C and VEGF-D. There are multiple isoforms of VEGF-A that result from alternative splicing of mRNA from a single, 8 exon VEGFA gene. These VEGF-A isoforms are denoted by their amino acid length, and include $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$. [194] $VEGF_{165}$ is the most abundant and potent isoform. Hypoxia inducible factor (HIF-1) is a key driver of VEGF, and under physiological conditions, stimulates VEGF release in response to tissue hypoxia. HIF-1 consists of 2 subunits, HIF-1α and HIF-1β. HIF-1α is a cytoplasmic protein and responsive to oxygen levels, and HIF-1β is a nuclear protein expressed independently of oxygen tension. In response to hypoxia, HIF-1α is stabilised and translocates to the nucleus, heterodimerises with HIF-1β forming an

active HIF-a protein. This protein binds to specific hypoxia response elements within the promotor region of hypoxia inducible genes, activating the transcription of VEGF. [195,196]

VEGF has multiple functions including as a growth factor for endothelial cells, a regulator of osteogenesis and mediator of platelet and leukocyte production. [197] VEGF excess causes microvascular permeability and endoneurial damage and therefore is thought to play a significant role in the clinical manifestations of extravascular volume overload, bone lesions, papilloedema, glomerular haemangiomata and arteriopathy in POEMS syndrome. [16] VEGF exerts its action through binding to high affinity type I transmembrane receptor tyrosine kinase receptors, VEGF receptor 1 and 2 (VEGFR 1 and 2). [16] Both receptors are responsible for the development and structural organisation of endothelial cells, including permeability resulting in the formation of transcellular gaps, vesiculovacuolar organelle formation and fenestrations. [197] VEGFR 1 is also highly expressed in macrophages implicating its unique role in the inflammatory response. [198] Interestingly VEGFR 2 receptors have been found to be present on neurones and Schwann cells in mice. [198] Previous studies have not demonstrated the direct effect of VEGF upon peripheral nerve endothelial cells, but histopathological evidence from sural nerve biopsies would support similar pathophysiological changes which are hypothesised to cause neuropathy in POEMS syndrome.

4.1.1.2 VEGF and plasma cells in POEMS syndrome

Understanding the mechanism which connects the monoclonal plasma cell dyscrasia of POEMS syndrome to the overproduction of VEGF is important to understanding pathways of disease. Wang *et al* demonstrated that POEMS syndrome plasma cells express high levels of VEGF mRNA and protein, the levels of which decline markedly following treatment [109]. Bone marrow plasma cell VEGF levels also correlate with serum, suggesting these plasma cells are the primary source of circulating VEGF in POEMS. This discovery was the first to definitively link the monoclonal gammopathy of POEMS syndrome with cytokine production. Interestingly polyclonal plasma cells secrete the greatest amount of VEGF, whereas the small populations of monoclonal cells which are thought to be the driver of disease in POEMS syndrome, are strongly IL-6 positive. Monoclonal cell IL-6 production may drive polyclonal plasma cell VEGF release. Since polyclonal plasma cells are more sensitive to chemotherapy this may explain the relative ease of obtaining complete VEGF suppression but less frequent complete haematological response due to resistance to treatment of the monoclonal cell population. The cases in which a clear monoclonal plasma cell population was present on bone marrow immunophenotyping were less likely to reach complete remission and experienced worse overall survival, supporting this theory. [109] An undiscovered question is whether such plasma cells secrete other proinflammatory cytokines that have been implicated in POEMS syndrome directly or whether they are produced elsewhere.

4.1.1.3 VEGF inhibition as POEMS therapy

The discovery of VEGF as a key mediatory of disease activity in POEMS syndrome prompted studies investigating the utility of VEGF blockade as a therapeutic option. This was of considerable research interest, as if successful, this would demonstrate VEGF as being the primary cytokine mediator responsible for causing pathogenesis in POEMS syndrome. Kuwabara et al reported treating six POEMS patients with the anti-VEGF antibody bevacizumab (avastin), monitoring VEGF levels and clinical response for outcome. [29] All patients had been previously treated unsuccessfully, with non-VEGF response and levels persisting between 600-4670pg/ml. Following bevacizumab, serum VEGF fell dramatically at 2 weeks to 46-190pg/ml and remained low at 8 weeks but relapsed in two patients as 12 weeks and 16 weeks in the remaining four. No clinical response was recorded in four patients, two of which subsequently died of multi-organ failure. Two patients showed a gradual improvement, but the effects were likely due to subsequent thalidomide therapy. A further 11 cases have been documented in the literature to have received bevacizumab, but all were used in combination therapies and thus the contribution of bevacizumab was unclear. [29,199,200] This treatment failure could be for several reasons. Firstly, suppressing VEGF would not influence the monoclonal plasma cells which have been demonstrated to be a potential source of VEGF expression and a driver of disease. Secondly, these data suggested other cytokines may have an important pathogenic role, and thus VEGF suppression alone would not switch off the multi-system activation of POEMS syndrome. An alternative explanation is that bevacizumab led to complete VEGF blockade, thus inhibiting the crucial physiological functions of VEGF (discussed above) leading to a number of downstream detrimental

effects. To note, deletion of both VEGF alleles in mice results in embryonic lethality, underscoring the importance of VEGF in human physiology. [197]

4.1.2 Alternative pro-inflammatory cytokines implicated in POEMS syndrome

Alterations in pro-inflammatory cytokines including fibroblast growth factor, hepatocyte growth factor, tumour necrosis factor, interleukin (IL) 6, IL-12, HIF1α and N-terminal propeptide of type I collagen have been reported in POEMS syndrome,[76,201,202] but more research is required to understand their roles in pathogenic mechanisms. A review of the literature of cytokines in POEMS syndrome was performed and the results summarised in table 4.1.

Table 4.1: Summary of research studies *measuring* cytokines in POEMS syndrome.

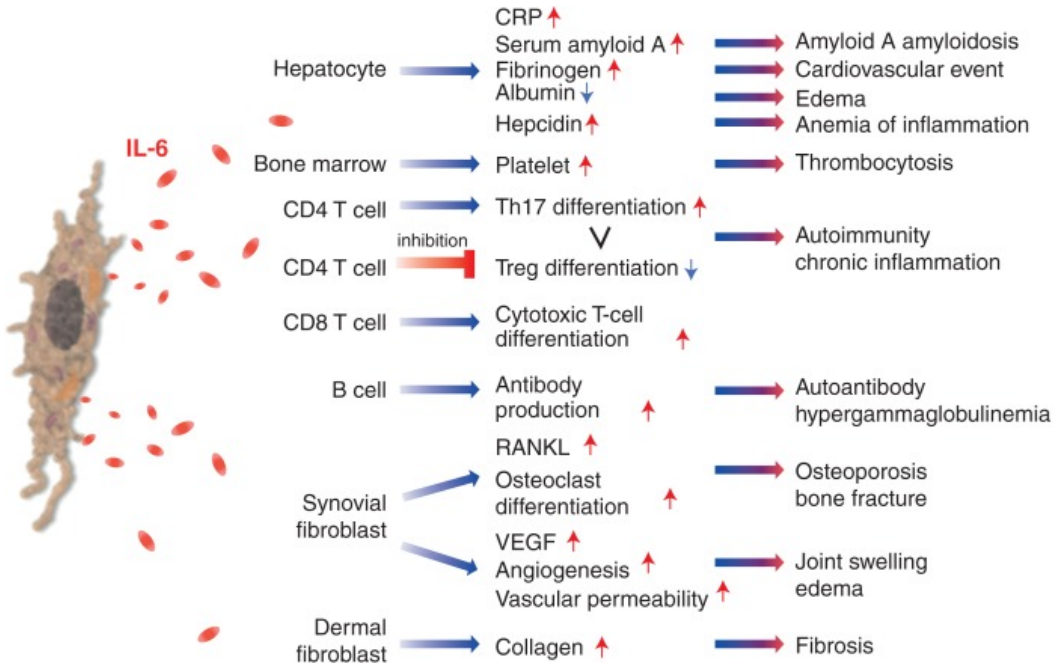
Paper title, year	Cytokines explored, patient groups	Findings	Biological relevance
Elevated serum interleukin-6 in POEMS syndrome reflects the activity of the disease, 1994 [77]	IL-6, IL-1β, TNF-α in 8 active vs 8 stable POEMS vs 40 other neurological disease	IL-6 increased in active POEMS. IL-1β not raised. No statistical difference in TNF-α.	IL-6 is an acute phase mediator and may underpin disease activity
Elevated Levels of Interleukin-1beta (IL-1β) and IL-6 in Serum and Increased Production of IL-1β mRNA in Lymph Nodes of Patients With POEMS Syndrome, 1994 [79]	IL-6, IL-1β in five POEMS patients vs 5 myeloma vs 5 Waldenstroms. In situ hybridization of mRNAs were performed on lymph nodes of 2 POEMS cases with Castlemans'.	Increased IL-1β (13/13) and IL-6 (7/13) in POEMS syndrome. Abundant IL-1β mRNA cells while IL-6 mRNA rare.	IL-6 is a B-cell differentiation factor which can induce antibody production and stimulate thrombopoiesis. IL-1β has fibrogenic and angiogenic properties.
Overproduction of Proinflammatory cytokines imbalanced by their antagonists in POEMS syndrome, 1996 [201]	IL-6, IL-1β, TNF-α, IL-2, interferon gamma and anti-inflammatory cytokines $TGF\beta_1$, IL-4, IL-10 and IL13. a2macroglobulin, IL-l receptor antagonist (IL-lra), soluble TNF receptors (sTNFr) p55 and p75, and soluble 11-6 receptor (slL-6r) in 15 POEMS vs 15 myeloma.	POEMS syndrome higher levels of IL-6 (in 10), IL-1β (in 14), TNF-α (in 10), and reduced levels of $TGF\beta_1$. IL-lra and sTNFrs compared to myeloma	Normal interferon and IL-2 suggest macrophage activation rather than T cells is primary process in POEMS syndrome. Speculation that light chain triggers monocytes/ macrophage production of cytokines. Antagonists i.e. $TGF\beta_1$ works as a macrophage deactivating factor. IL-1ra and sTNFrs insufficient to buffer the effects of increased cytokine production.
POEMS syndrome: report on six patients with unusual clinical signs, elevated levels of cytokines, macrophage	IL-6, IL-1β, TNF-α in six POEMS cases vs five myeloma vs three neuropathy + plasma dyscrasia vs four MGUS. Ganglioside	Mod to marked increase IL-6, TNF-α. mRNAs on lymph nodes showed strong positivity for IL-1β. Skin biopsy high	TNF-α has a role in polyneuropathy. High levels in GBS and paraproteinemic neuropathy/ autoimmune demyelination through altering the blood nerve barrier.

involvement and chromosomal aberrations of bone marrow plasma cells, 1997 [20]	antibodies In situ hybridisation of DNA for TNF and IL-1β	number of cells expressing TNF-α	IL6 and TNF-α both macrophage produced and thus POEMS is due to macrophage activation rather than T cell.
Circulating levels of MMP-1, -2, -3, -9, and TIMP-1 are increased in POEMS syndrome, 2001 [76]	MMP-1,-2,-3, -9 and TIMP-1 n 15 POEMS vs 18 other neurological disorders (OND) and controls	MMP-1,-2, -3, -9 and TIMP 1 increased in POEMS. VEGF and TIMP-1 correlated.	MMP involved in immune mediated demyelinating neuropathies including GBS and CIDP. MMP-9 and MMP-3 increased early in course of EAN. MMP-9 detected in Schwann cells and endoneurial vessels. Postulate MMP-2 and -9 degrade collagen of blood nerve barrier, then MMP-2, -3 and -9 degrade myelin basic protein.
Polyneuropathy in POEMS syndrome: role of angiogenic factors in the pathogenesis, 2005 [16]	VEGF and EPO in 11 patients in serum correlated with VEGF and EPO peripheral nerve expression and degree of endoneurial vessel involvement.	POEMS associated with high VEGF, low EPO. POEMS nerves VEGF highly expressed in blood vessels and some non-myelin forming Schwann cells. EPO and EPO-R localised to nerve vasculature and expressed similar to normal. Increased thickening of basal lamina and narrowing of lumen of endoneurial vessels in POEMS with endoneurial vessel proliferation.	Angiogenic factors are diagnostic, pathogenic and prognostic markers of POEMS. VEGF leads to microvascular hyperpermeability induced by VEGF opening blood nerve barrier. Endothelial injury occurs due to VEGF. Nerve hypoxia leads to increased HIF-1α and secondary local VEGF expression causing toxic gain of function. EPO typically neuro protective and thus reduced levels contributes to neuropathy.
Markedly upregulated serum interleukin-12 as a novel biomarker in POEMS syndrome, 2012 [192]	A 27-cytokine assay in 16 untreated POEMS vs 6 relapsed vs 10 paired pre and post treatment POEMS vs 10 CIDP vs 19 healthy controls.	IL-1β, IL-6, IL-7, IL-8, IL-10, Il-12, Il-13, Il-17, GM-CSF, MIP-1 α, VEGF and TNF-α. IL-12 and VEGF markedly increased above reference ranges. Post treatment IL-12 and VEGF decrease, IL-6 and TNF-α were higher post treatment than pre (not	There is a cytokine storm in POEMS syndrome, typified by IL-12 and VEGF. IL-12 is proinflammatory and produced by monocytes, macrophages and B-cells. Involved in early phase of immune response and differentiation of Th1 cells. IL-12 injected in rat peripheral nerves cause demyelination. IL-12

		significant).	increases in GBS, therefore IL-12 may contribute to pathogenesis of demyelination in POEMS.
Multiple angiogenetic factors are upregulated in POEMS syndrome, 2013 [78]	Basic fibroblast growth factor (bFGF) and HGF in 17 POEMS patients.	Higher level of bFGF and HGF in POEMS. Post bevacizumab, VEGF but not HGF dropped suggesting independently released.	bFGF is upstream of and regulates VEGF and HGF. bFGF not correlated with VEGF suggesting upregulation is not VEGF influenced. bFGF enhances function of blood nerve barrier by producing claudin 5 so may be released as feedback to VEGF.
Markedly elevated serum total N-terminal propeptide of type I collagen (P1NP) is a novel marker for the diagnosis and follow up of patients with POEMS syndrome, 2014 [202]	P1NP in 45 POEMS patients vs 28 healthy controls vs 18 CIDP vs 11 CD vs 15 myeloma vs 10 AL amyloid.	P1NP markedly elevated in POEMS compared to normal and disease matches. ROC shows P1NP cut off of 70ng/ml for POEMS with sensitivity 80%, specificity 91.5%. No statistical difference was observed between P1NP and VEGF for diagnostic performance.	N-terminal propeptide of type I collagen (P1NP) is a marker of bone formation and is a useful biomarker of POEMS disease activity. Non-specific, raised in myeloma and bone metastases.
Pentraxin-3 and VEGF in POEMS syndrome: A 2-year longitudinal study, 2014 [203]	Pentraxin-3 in 6 POEMS patients vs 16 controls measured across 2 years. Expression of pentraxin-3 also assessed on sural nerve biopsies from POEMS vs vasculitis patients.	No correlation between pentraxin-3 and VEGF in POEMS. Sural nerve biopsies from vasculitis patients and not POEMS showed pentraxin-3 staining.	Pentraxin is involved in vessel inflammation, reflecting vessel injury and repair. Is not involved in POEMS syndrome.

To summarise from the table above, IL-6 is the cytokine most consistently cited to be raised in active POEMS syndrome compared to disease matched cases and healthy controls. IL-6 was first discovered in 1986 for its role in stimulating B cells to produce immunoglobulin G (IgG), now recognised as a multifunctional cytokine that regulates numerous biological processes such as inflammation, haematopoiesis and metabolism. [204] Dysregulated IL-6 has been demonstrated to underlie a number of autoimmune, malignant, inflammatory and neuroinflammatory conditions. It also induces activity of acute phase proteins such as CRP, serum amyloid A and fibrinogen, and has been shown to stimulate VEGF production from both nonimmune cells such as fibroblasts, and from plasma cells. [205,206] The host of IL-6 mediated effects inducing systemic inflammation, clotting, bone abnormalities and B-cell stimulation appear consistent with several key features of POEMS syndrome.

Figure 4.1: IL-6 in inflammation, immunity and disease.



Taken from IL-6 in inflammation, immunity and disease. [205] *Treg, regulatory T cell; RANKL, receptor activator of nuclear factor kB ligand.*

IL-1β has been implicated in a range of autoinflammatory diseases and metabolic syndromes, and found to be raised in most, but not all, studies in POEMS syndrome (table 4.1). Of possible relevance, IL-1β also has a role in central and peripheral neuropathic pain. [207] IL-1 receptor 1 (IL-1R1) is expressed in sensory neurones, and IL-1β is known to modulate neuronal excitability in neurones through these receptors and affect their interactions with sodium channels, GABA and NMDA receptors. [208] In animal models of neuropathic pain, peripheral nerve injury leads to Schwann cell activation and recruitment of macrophages, both of which secrete IL-1β. In addition, injections of IL-1R1 neutralising antibodies were shown to reduce both thermal hyperalgesia and mechanical allodynia in a chronic sciatic nerve constriction pain injury in rats. [209] Distal, cramping neuropathic pain is a common characteristic of POEMS syndrome, reported in 75% of our cohort. The quality of pain is distinct, and different from other causes of immune mediated inflammatory neuropathy other than those resulting in vasculitis, amyloidosis or cryoglobulinemia, and could be an influence of cytokine driven neuronal excitability.

TNF-α also appears implicated in POEMS syndrome, and has been proposed to specifically contribute to the polyneuropathy. This was based on studies which demonstrated high levels of TNF-α in other demyelinating neuropathies, notably GBS, but also paraproteinemic neuropathies. GBS is characterised by activated T cell and macrophage infiltration in peripheral nerves which are likely key components of the immune mediated response. Both cell types secrete TNF-α which probably have toxic effects on myelin, Schwann, and endothelial cells. [210] Alterations in endothelial homeostasis result in an increase of vascular permeability and breakdown of the blood

nerve barrier, potentially exposing neurones to further cytokine and immune mediated toxicity .[210] Although somewhat speculative in peripheral nervous system biology, this phenomenon has been demonstrated in animal studies of the blood brain barrier (BBB), where intracisternal injection of TNF-α in rats resulted in increased permeability of the barrier and neutrophil infiltration.[211] Similar results have also been seen *in-vivo* in multiple sclerosis and bacterial meningitis, where high levels of TNF-α in the CSF and serum correlate with the CSF to serum albumin ratio, a marker of blood nerve barrier permeability, although other factors may contribute.[212,213]

Of interest, IL-6, IL-1β and TNF-α in combination have been seen to induce VEGF production in synovial fibroblast cultures, indicating that co-stimulation of several cytokines may be required. VEGF production was not induced when each cytokine was studied in isolation.[214] If similar biological processes underpin VEGF production in POEMS syndrome, it may be that targeting individual cytokines (such as using Tocilizumab to inhibit IL-6) may be considered an alternative strategy to impair VEGF production other than total blockade with Bevacizumab, which had unsuccessful outcomes.

Although the previously reported range of cytokine studies are a compelling insight to POEMS pathogenesis, it must be noted that the cytokines identified were likely studied because of the availability of suitable assays, and thus do not provide the full picture of the cytokine network of POEMS syndrome. The most comprehensive cytokine analysis to date has been by Kanai et al (2012), who studied 27 cytokines using a Bio-Plex

human 27-Plex cytokine panel kit (Bio-Rad, Hercules, CA) in 16 untreated, 6 relapsed and 10 post treated POEMS patients, compared to 10 CIDP cases and 19 healthy controls. IL-1β, IL-6, IL-7, IL-8, IL-10, Il-12, Il-13, Il-17, GM-CSF, MIP-1 α, VEGF and TNF-α were all significantly raised in POEMS compared to controls, but only IL-12p70 and VEGF dropped significantly following therapy, suggesting their potential importance in POEMS pathology.[192] The authors cite that IL-12p70 is produced primarily by antigen presenting cells and plays a primary role in the induction of cell-mediated immunity. The authors additionally consider IL-12 as being potentially neurotoxic, citing a number of studies which have demonstrated demyelination through intraneural injection. There is uncertainty regarding the true functional role of IL-12. Other studies have demonstrated IL-12 being involved in anti-tumour immune responses, activating natural killer cells and CD8+ cytotoxic T lymphocytes, leading to interferon gamma production, which has a role in preventing angiogenesis, tumour initiation, growth and metastasis.[215] This also suppresses the production of VEGF, and therefore may be considered to be a regulatory cytokine produced in response to systemic inflammation rather than part of the initial proinflammatory cascade.

Identification of the cytokines raised or suppressed in POEMS syndrome will enable deeper understanding of the immunological networks which drive pathogenesis. Knowledge of cytokines' function in health and disease may uncover the role they play, and the downstream physiological processes which take place leading to disease. For this reason, the following chapter addresses the following hypotheses and aims to support or refute these assumptions.

4.1.3 Hypotheses

1. POEMS syndrome is a cytokine mediated disease.
2. Sera of patients with POEMS syndrome exhibit a unique cytokine fingerprint which underlies the pathogenic mechanism of disease, possibly including some cytokines which drive neuropathy.
3. Such cytokines may serve as biomarkers of disease severity
4. Such biomarkers are supressed following successful treatment

4.1.4 Aims

1. Test sera of POEMS patients for a range of potentially pathogenic cytokines
2. Identify cytokines which are raised in POEMS syndrome compared to similar haematological and neuropathy causing diseases, and healthy controls
3. Establish cytokines which respond to treatment.
4. Correlate cytokines with other markers of disease.

4.2 Methods

4.2.1 Study participants

Participants recruited to this study were selected from the UK POEMS database. The inclusion criteria of pre-treatment, post-treatment and relapsed POEMS syndrome cases are defined in 3.5.2.1.

Patients with Chronic Inflammatory Demyelinating Neuropathy who fulfilled the EFNS/PNS diagnostic criteria were included as neurological controls. Patients were classified based on the CIDP Disease Activity Status (CDAS), [216] and only patients with a CDAS score of 5 were selected for study, that is patients with newly diagnosed or unstable active disease.

Patients with a new presentation (i.e. untreated) of secretory multiple myeloma, which fulfilled the International Myeloma Working Group Criteria for the diagnosis of multiple myeloma, were selected as haematological controls. The underlying haematological dyscrasia in POEMS and myeloma is similar, although myeloma does not itself cause neuronal damage, and thus different cytokine profiles between the two were thought to potentially allude to neuropathic markers. Any monoclonal protein composition was acceptable for inclusion.

Twenty healthy control cases were selected so the number of POEMS: non-POEMS cases were similar. All cases were matched for age and sex.

4.2.2 Sample collection and processing

Blood was collected into serum tubes, centrifuged at room temperature at 3000 rpm for 5 minutes. Serum was separated and aliquoted immediately, then frozen at -80 oC until use.

4.2.3 Cytokine assay

Serum concentration of 30 cytokines, chemokines and proinflammatory molecules were measured using the MSD V-PLEX Human Cytokine 30-Plex kit (Meso-Scale Diagnostics, Rockville, MD, U.S.A). This kit consists of the cytokines Granulocyte-macrophage colony stimulating factor (GM-CSF), Interleukin (IL) IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, tumour necrosis factor-beta (TNF-β), vascular endothelial growth factor- A (VEGF-A), the chemokines eotaxin, macrophage inflammatory protein-1 beta (MIP-1β), eotaxin-3, thymus- and activation-regulated cytokine (TARC), interferon γ-induced protein 10 (IP-10), MIP-1α, IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage derived chemokine (MDC), MCP-4, and the proinflammatory molecules interferon gamma (IFN-γ), IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α.

In short, 50ul of prepared samples, calibrators or controls were added to each well, sealed and incubated at room temperature with shaking for two hours. Following three washes with PBS+0.05% Tween-20, 25ul of detector antibody solution was added to each well, sealed and incubated at room temperature for two hours. Following three

further washes, 150ul of 2x read buffer was added to each well, and the electrochemiluminescent signal (ECL) was detected by a MESO Quickplex SQ 120 plate reader (MSD) and analysed with Discovery Workbench Software (v4.0, MSD).

4.2.4 Statistical analysis

Tests of normality were performed on cytokine levels by Shapiro-Wilk test. Kruskal-Wallis tests were used to compare the pre-treatment POEMS syndrome, CIDP, myeloma and healthy control groups to first identify groups in which statistically significant differences existed. If deemed significant (that is, a significant difference between all groups has been identified, but does not signify which individual groups differ), post-hoc testing with Dunn's test was performed comparing POEMS pre-treatment vs CIDP, POEMS pre-treatment vs myeloma and POEMS pre-treatment vs healthy control independently to ascertain whether a significant difference existed between individual groups. Dunn's test corrects for multiple comparisons by adjusting the significance threshold (i.e the value of alpha) based on the number of comparisons. Comparing preselected variables in this case (rather than comparing each group against one another) is appropriate to the research question, but also reduces the overall number of statistical comparisons being performed, which increases power. Cytokines which significantly differed in pre-treatment POEMS syndrome compared to all other conditions were considered to be most relevant. We defined the upper and lower limits of normal of each cytokine as the mean ±3 SD of normal healthy control values.

Cytokines identified as being relevant in POEMS syndrome were compared to other cytokines and to clinical features such as degree of plasma cell infiltration (on bone marrow), difference in free light chain level, neuropathy severity using the Overall Neuropathy Limitation Score, and prognostic risk score using Spearman's rank correlation.

Binomial Logistic regression analysis was used to compare VEGF alone as the 'gold standard' diagnostic biomarker in POEMS syndrome compared to or in combination with a number of identified relevant cytokines, to identify which model most accurately identifies POEMS cases from non-POEMS (from the entire cohort including CIDP, myeloma and healthy controls). Receiver operating characteristic (ROC) analysis was also used to determine each model's area under the curve (AOC), and the positive and negative predictive power. To ascertain whether cytokines could potentially predict the degree of haematological disease, univariate regression analysis was performed for each potentially pathogenic cytokine against paraprotein level, SFLC ratio, difference in free light chains and % plasma cells on bone marrow biopsy through linear regression analysis. Univarate analysis was also then performed to see whether cytokine levels could predict functional outcome against the ONLS score, and likelihood of progression or death using logistic regression. If significant, a forward selection stepwise regression model would be performed to identify potential confounding variables and control for them in a model.

Biomarkers were then analysed to determine their response to therapy and/ or relapsed disease (behaving as a marker of disease activity). POEMS pre-treatment cases in which matched (i.e. of the same patient) post-treatment sera were available (n=38) were selected and cytokines compared using Wilcoxon signed-rank test. Of the 10 samples in which matched relapse sera (i.e. of the same patient) were additionally collected, post-treatment cytokine levels were compared to relapsed sera by Wilcoxon signed-rank test. To determine whether the degree of cytokine suppression following treatment determined the possibility of relapse, post-treatment cytokine levels were compared between patients who remained disease free (n-28), and those who have relapsed (n=10) using Mann-Whitney tests (independent samples of differing group size).

4.3 Results

4.3.1 Study participants and samples

Sixty-eight POEMS cases were included in the study. There were 68 pre-treated samples, of which 38 had matched post treatment serum, and 10 had pre, post and relapsed sera. Patient demographics and clinical characteristics are included in table 4.2. Median age of the pre-treated POEMS cohort was 54 years (IQR 45-68) with male to female ratio of 43:25. Clinical features, paraprotein distribution, complications and therapies were broadly similar to that of the entire POEMS cohort study characteristics described in chapter 2. Table 4.2 describes the clinical features of the POEMS cohort overall (pre-treatment cases n=68), and of sub-groups in which analyses were conducted such as those in which post treated sera (n=38), and relapsed sera (n=10) were available. Characteristics of the post-treatment and relapsed cohorts did not differ

significantly from that of the entire pre-treatment cohort, demonstrating these smaller groups were representative. Of the CIDP cohort, 14 were new diagnoses and treatment naïve, and six patients were being treated with IVIg and were clinically deteriorating. All patients were therefore considered to have 'unstable active disease' as per the CIDP disease activity scale, with a score of 5. CIDP patients' median age was 56 (IQR 48-68) with a male to female ratio of 13:7. Of the myeloma cohort, six had IgG kappa clonal disease, four IgA kappa, five IgG lambda, two IgA lambda, 2 kappa light chain disease and one lambda light chain. Myeloma patients' median age was 65 (IQR 53-74) with a male to female ratio of 12:8. The healthy control group median age was 58 (IQR 51-70) with a 12:8 male to female ratio. Thus, all samples were appropriately matched for age and sex distribution. All samples analysed were collected between 2015 and 2020 and stored at a maximum of -20.

Table 4.2: POEMS cohort demographics and clinical characteristics

	POEMS pre (n=68)	POEMS post matched (n=38)	POEMS relapsed (n=10)
Sex m:f (%m)	43:25 (63)	20:18 (57)	6:4 (60)
Median age, y (IQR)	54 (45-68)	55 (45-69)	47.5 (35.75-56.50)
Alive: dead	61:7	37:1	10:0
Symptoms to diagnosis, m (IQR)	10.5 (6-23.75)	10 (6:21)	9.5 (5.75-14.25)
Diagnostic features (n, %)			
Polyneuropathy	65 (96)	38 (100)	10 (100)
Monoclonal	68 (100)	38 (100)	10 (100)
Bone lesions	54 (79)	31 (82)	9 (90)
Castlemans	10 (14)	4 (11)	1 (10)
Organomegaly	31 (45)	14 (37)	4 (40)
Volume O/L	59 (87)	32 (84)	7 (70)
Endocrinopathy	37 (54)	18 (47)	6 (60)
Skin lesions	48 (70)	27 (71)	7 (70)
Papilloedema	28 (41)	15 (40)	4 (40)
Polycythaemia	11 (16)	6 (16)	1 (10)
Thrombosis	23 (34)	13 (34)	4 (40)
Thrombocytosis	28 (41)	12 (32)	3 (30)
Paraprotein on SPE (n,%)	40 (59)	20 (52)	5 (50)
Immunofixation IgA*	29 (43)	15 (40)	3 (30)
Immunofixation IgG	23 (34)	15 (40)	5 (50)
Not detectable	16 (23)	8 (20)	2 (20)
Abnormal SFLC	9 (13)	7 (18)	1 (10)
dFLC >10	28 (41)	17 (44)	4 (40)
Bone marrow plasma cell %			
0%	27 (40)	19 (50)	4 (40)
1-5%	24 (35)	15 (40)	5 (50)
6-10%	8 (12)	3 (8)	1 (10)
>10%	9 (13)	1 (2)	0 (0)
Low albumin	16 (24)	7 (18)	1 (10)
Low eGFR	34 (50)	17 (44)	1 (10)*
Median ONLS score (IQR)	6 (3.25-8)	6 (3.25-8)	7.5 (4.75-9)
Stroke	7 (10)	4 (11)	1 (10)
Myocardial infarction	5 (7)	5 (13)	3 (30)
Pulmonary hypertension	32 (47)	17 (45)	4 (40)
Treatments (first line)			
Radiotherapy	6 (9)	5 (7)	
Cyclophosphamide	2 (3)	0 (0)	2 (20)
Lenalidomide	35 (51)	18 (26)	0 (0)
ASCT	25 (37)	15 (22)	1 (10)*

IQR = interquartile ratio, SPE= serum protein electrophoresis, dFLC= difference in free light chain (kappa – lambda), eGFR= estimated glomerular filtration rate.
**= significantly differ compared to POEMS pre group (p<0.05)*

4.3.2 Cytokine profiles of POEMS syndrome compared to disease matched and healthy control groups.

Firstly, 30 cytokines from 68 POEMS cases, 20 CIDP, 20 Myeloma and 20 healthy controls were compared and analysed. Table 4.3 displays the cytokine profiles across the 5 groups, which of those that were significantly different across groups (ie ANOVA of $p<0.05$), and whether significantly different cytokine levels existed between POEMS syndrome patients compared to CIDP, myeloma and healthy controls. Of the cytokines analysed, 15 cytokines exhibited significant differences between the POEMS cohort compared to the other groups. Serum levels of the following cytokines were significantly raised in the POEMS group: VEGF, IL-7, IL-16, IL-6, IL-4, MIP-1α, Eotaxin-3, IL-15, IL-5, MCP-1, TNF-α, and MIP-1β (see table 4.3). Note that levels of IL-4 in the myeloma group were recorded under the limit of quantification at 0.01pg/ml and thus may not be accurate or quantifiable, however were significantly raised in both POEMS and CIDP. Levels of cytokines which were markedly elevated in POEMS syndrome compared to all other groups, and above the calculated upper limit of normal were VEGF (mean 2376 pg/ml, ULN 338 pg/ml) , IL-7 (34.70 pg/ml, ULN 28.39 pg/ml), IL-16 (426 pg/ml, ULN 388 pg/ml) and IL-6 (2.34 pg/ml, ULN 1.64 pg/ml). Serum levels of IP-10, MDC and Eotaxin were significantly reduced in POEMS syndrome compared to other groups, but less uniform across groups. IL-10 for example was reduced in POEMS syndrome compared to other disease groups, but similar to that of healthy controls. MDC was significantly reduced compared to both CIDP and healthy controls, but similar to myeloma, and Eotaxin was reduced only when compared to that of myeloma.

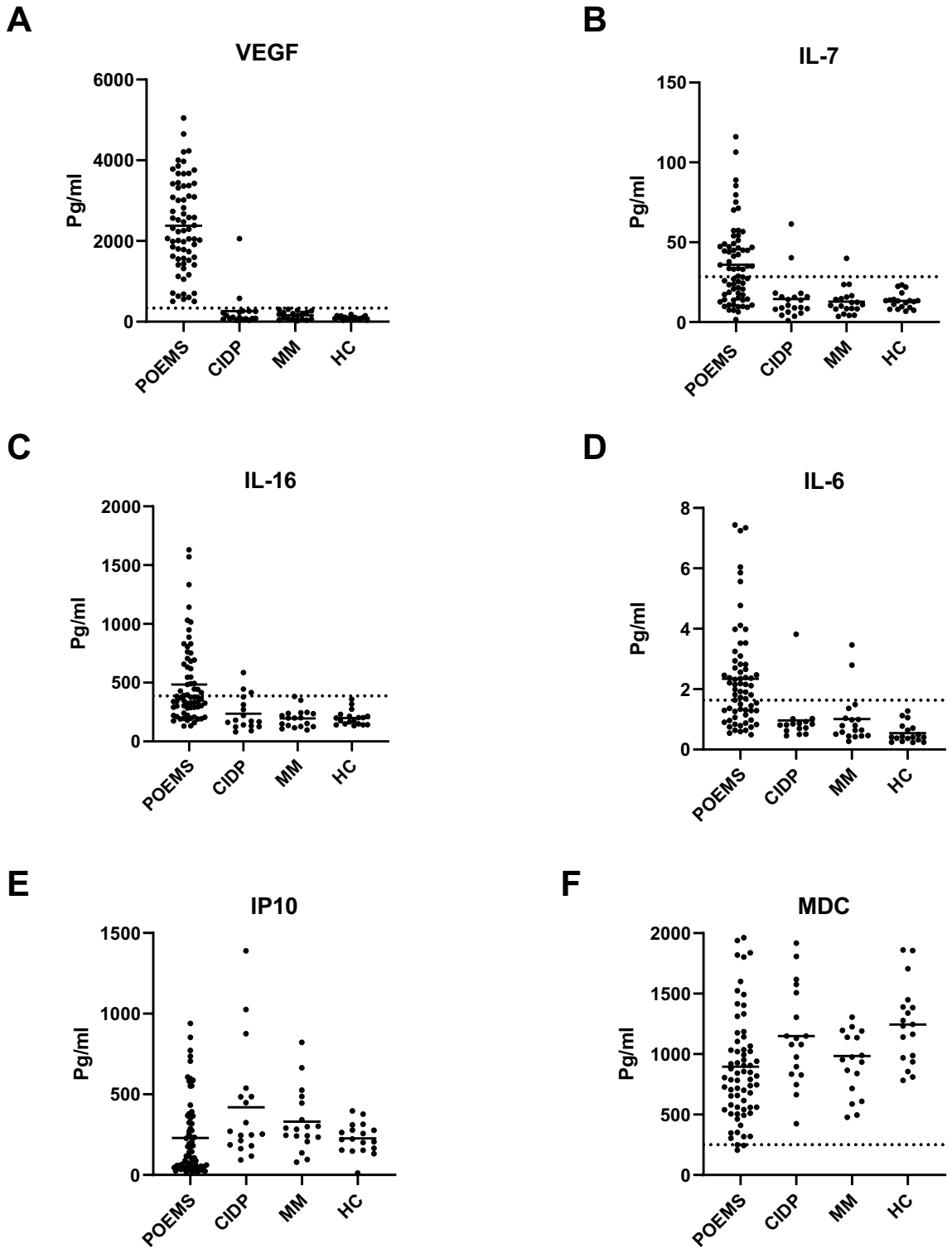
Table 4.3 Summary of serum cytokine levels in POEMS syndrome, CIDP, multiple myeloma and healthy controls.

	ANOVA p-value	POEMS syndrome (n=68) pg/ml Mean (CI)		CIDP (n=20) pg/ml Mean (CI)		**POEMS vs CIDP**	Myeloma (n=20) pg/ml Mean (CI)		**POEMS vs Myeloma**	Healthy controls (n=20) pg/ml Mean (CI)		**POEMS vs Healthy controls**
Increased in POEMS												
VEGF	**<0.0001**	2376	(2105-2647)	158	(87.6-228)	****	155	(106-205)	****	92.8	(73.2-112)	****
IL-7	**<0.0001**	34.7	(29.1-40.2)	10.3	(7.78-12.9)	****	11.3	(8.53-14.1)	****	13.3	(10.9-15.6)	***
IL-16	**<0.0001**	426	(367-484)	235	(161-310)	***	196	(156-236)	****	190	(164-216)	****
IL-6	**<0.0001**	2.34	(1.93-2.75)	0.964	(0.516-1.41)	***	1.00	(0.586-1.43)	***	0.544	(0.385-0.704)	****
IL-4	**<0.0001**	0.037	(0.027-0.048)	0.036	(0.020-0.051)	NS	0.007	(0.005-0.009)	****	0.012	(0.006-0.018)	***
MIP-1α	**<0.0001**	83.0	(26.0-140)	91.9	(-2.81-186)	NS	29.9	(2.13-57.7)	*	13.1	(11.4-14.7)	****
Eotaxin-3	**<0.0001**	17.7	(15.0-20.5)	22.6	(14.8-30.3)	NS	9.15	(7.08-11.2)	**	9.92	(7.03-12.8)	*
IL-15	**<0.0001**	3.38	(3.05-3.70)	2.62	(2.27-3.02)	NS	4.03	(3.22-4.84)	NS	2.21	(2.01-2.42)	***
IL-5	**<0.005**	0.347	(0.273-0.422)	0.675	(0.377-0.972)	NS	0.435	(0.191-0.674)	NS	0.122	(0.077-0.168)	*
MCP-1	**<0.01**	326	(283-368)	192	(138-247)	**	243	(198-289)	NS	232	(197-267)	NS
TNF- α	**<0.05**	3.71	(3.16-4.26)	3.13	(2.35-3.91)	NS	3.11	(2.29-3.93)	NS	2.17	(1.76-2.57)	*
MIP-1β	**<0.05**	260	(139-381)	227	(126-328)	NS	195	(77.5-312)	NS	113	(85.4-141)	*
Decreased in POEMS												
IP-10	**<0.0005**	182	(138-226)	361	(228-495)	**	330	(234-426)	**	227	(180-274)	NS
MDC	**<0.005**	896	(784-1008)	1149	(947-1351)	*	984	(797-1173)	NS	1244	(1079-1409)	**
Eotaxin	**<0.05**	233	(201-266)	297	(217-377)	NS	392	(294-489)	*	316	(233-399)	NS

Unchanged in POEMS												
GM-CSF	NS	0.112	(0.08-0.15)	0.124	(0.06-0.14)	NS	0.061	(0.035-0.087)	NS	0.066	(0.049-0.083)	NS
IL-12p40	NS	175	(146-203)	165	(120-210)	NS	140	(102-179)	NS	144	(117-172)	NS
IL-12p70	NS	0.321	(0.252-0.390)	0.324	(0.185-0.436)	NS	0.184	(0.103-0.264)	*	0.209	(0.134-0.284)	NS
IL-17a	NS	1.96	(1.61-2.32)	2.65	(1.76-3.53)	NS	1.65	(1.24-2.05)	NS	1.80	(1.51-2.10)	NS
IL-1α	NS	1.05	(0.852-1.26)	3.10	(1.45-4.74)	*	0.827	(0.015-1.68)	NS	0.666	(0.384-0.948)	NS
TNF-1β	NS	0.136	(0.092-0.178)	0.129	(0.086-0.172)	NS	0.153	(0.113-0.193)	NS	0.164	(0.114-0.179)	NS
IL-8	NS	768	(336-1201)	780	(353-1208)	NS	336	(56.9-616)	NS	672	(17.8-1326)	NS
MCP-4	NS	180	(149-211)	140	(77.9-203)	NS	119	(75.6-162)	NS	121	(105-137)	NS
TARC	NS	281	(232-330)	334	(169-498)	NS	345	(227-464)	NS	501	(320-683)	*
INF- γ	NS	5.74	(4.41-7.08)	7.18	(4.49-9.87)	NS	6.41	(3.13-9.66)	NS	3.77	(2.63-4.83)	NS
IL-10	NS	0.793	(0.545-1.04)	0.521	(0.297-0.745)	NS	1.36	(0.108-2.62)	NS	0.515	(0.225-0.806)	NS
IL-13	NS	0.943	(0.675-1.21)	1.73	(0.984-2.47)	*	0.864	(0.515-1.21)	NS	0.818	(0.604-1.03)	NS
IL-1β	NS	0.484	(0.234-0.735)	0.452	(0.137-0.768)	NS	0.188	(0.111-0.266)	NS	0.151	(0.954-0.207)	NS
IL-2	NS	0.432	(0.288-0.512)	0.373	(0.231-0.514)	NS	0.313	(0.199-0.427)	NS	0.521	(0.179-0.86)	NS

*VEGF= Vascular endothelial growth factor, IL= interleukin, MIP= macrophage inflammatory protein, MCP= monocyte chemoattractant protein ,TNF= tumour necrosis factor, IP= interferon γ-induced protein MDC= macrophage derived chemokine, GM-CSF= Granulocyte-macrophage colony stimulating factor, TARC= thymus- and activation-regulated cytokine, INF= interferon. P -value set at <0.05. NS= not significant, *=<0..05, **<0.005, ***<0.0005 , ****<0.0001.*

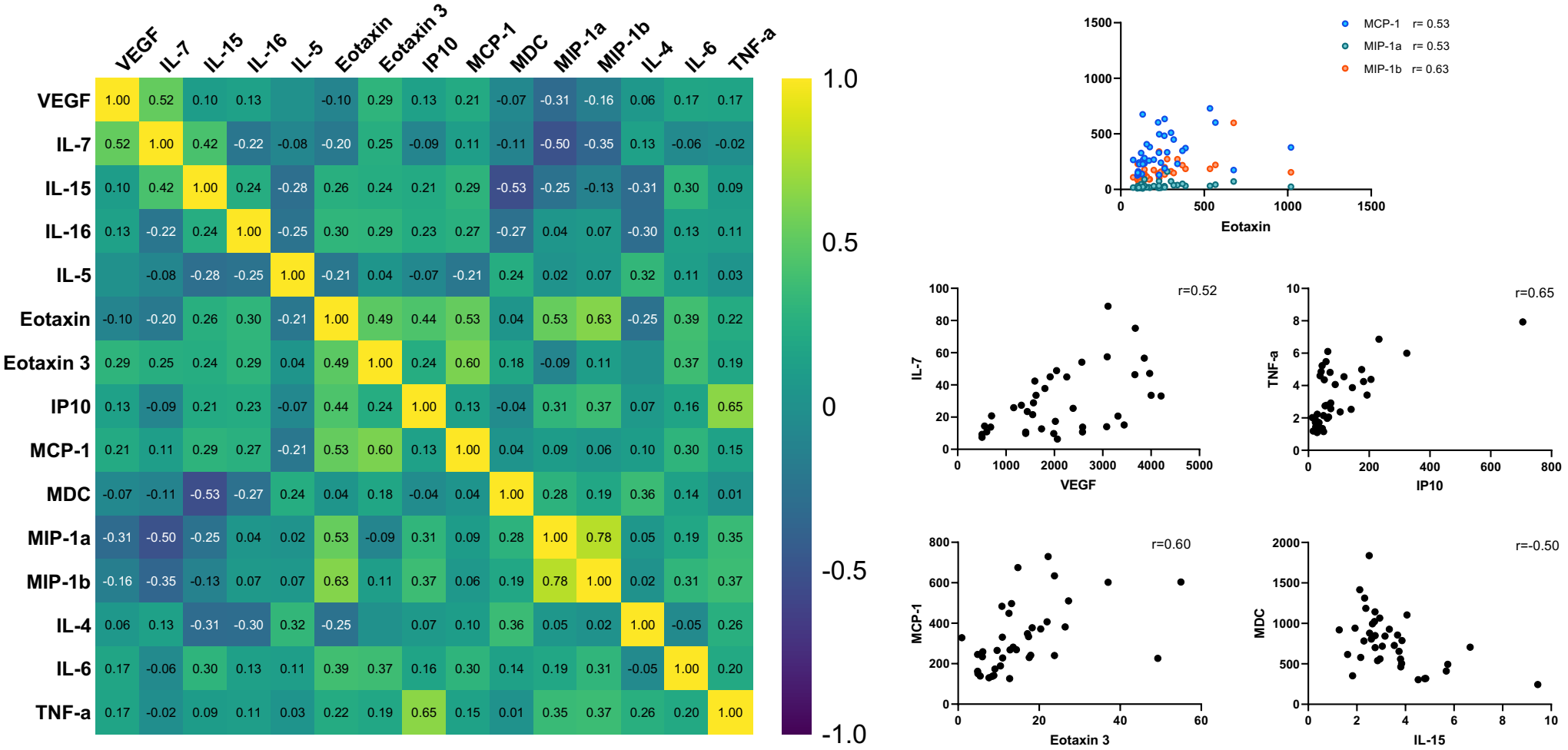
Figure 4.2: Cytokine levels in pre-treatment POEMS cases compared to CIDP, multiple myeloma and healthy control



Serum levels of VEGF (A), IL-7 (B), IL-16 (C) , IL-6 (D), IP-10 (E) and MDC (F). Dashed line indicates upper or lower limit of normal (mean ±3 SD of normal healthy control values. Horizontal lines represent median cytokine values.

A Spearman correlation matrix was performed to determine whether potentially relevant pre-treatment cytokines in POEMS syndrome correlate with one another, which may provide more evidence towards their presence in active disease. Relevant cytokines were identified as those which significantly differed in POEMS compared to other disease groups. Of the cytokines analysed, the cytokine which correlated with VEGF most strongly was IL-7 ($r=0.52$, $p=0.008$). No other cytokines were shown to correlate with VEGF. The other cytokines which correlated with an r value >0.5 were Eotaxin with MCP1, MIP-1a and MIP-1b, Eotaxin-3 with MCP-1 and IP-10 with TNF-α. Il-15 was inversely correlated with MDC. VEGF and IL-7, Eotaxin and MCP1, IP-10 and TNF-α and MIP1a and MIP1b continued to display correlation in post-treatment sera, potentially alluding to cytokine pathways or inherent relationships between cytokines which define the POEMS inflammation fingerprint. For example, although IL-7 and VEGF have not been demonstrated to be interdependent in any way, they correlate with one another even following therapy, which likely indicate they both independently reflect underlying disease activity.

Figure 4.3: Cytokine correlations in POEMS syndrome



Correlation matrix depicting strongest positive associations in yellow, strongest negative in purple (left). Graphs depicting cytokine correlations of r>0.5 (p<0.05) (right.

4.3.3 POEMS syndrome cytokines as diagnostic biomarkers of disease

The diagnostic utility of VEGF is well known and has been reported earlier in this thesis. The four cytokines which were significantly raised in POEMS syndrome compared to all other disease categories and healthy controls were VEGF, IL-7, IL-16 and IL-6. Univariate logistic regression was initially performed to compare the predictive ability of each cytokine for the diagnosis of POEMS syndrome, looking at results across the entire cohort of POEMS, CIDP, myeloma and healthy control values, the results of which are displayed in table 4.4. Multivariate logistic regression was used to then compare whether a combination of VEGF, IL-7, IL-16 and IL-6 was more accurate at diagnosing POEMS syndrome than VEGF alone, in both circumstances using a cut off value of 0.5 which in both cases gave the greatest sensitivity and specificity. This analysis deemed the combination of four cytokines as demonstrating greater predictive ability for a correct diagnosis ($p=0.0002$), with an area under the ROC cure (AOC) of 0.997 ($p<0.0001$), negative predictive power of 94.4% and positive predictive power of 98.5%. VEGF independently also however demonstrates high diagnostic predictive power, with an AOC of 0.9901, negative predictive power of 92.7% and positive of 98.4%.

Table 4.4 *Univariate p*redictive ability of pre-treatment cytokine levels and diagnosis of POEMS syndrome

Univariate analysis for diagnosis of POEMS				
Variable	**OR**	**95% CI**	**p**	**AOC**
VEGF	1.006	1.004-1.009	<0.001	0.99
IL-7	1.087	1.053-1.130	<0.001	0.81
IL-16	1.002	1.000-1.004	0.0237	0.82
IL-6	5.520	2.933-12.97	<0.0001	0.87

Univariate regression analysis of the four cytokines (VEGF, IL-7, IL-16 and IL-6) demonstrated that none of the cytokines could be used to accurately depict indices of the haematological monoclonal plasma cell disease, functional outcome as per the ONLS or predict patients who progressed or died. This is similar to the regression model analysis in chapter 2 which only looked at VEGF as a biomarker, using a different VEGF assay (R&D systems) and included the entire 100 patient POEMS cohort. Again in this circumstance, total VEGF levels at presentation did not predict the likelihood for progression or death, although post treatment VEGF response (i.e. if normalised or not) did.

4.3.4 POEMS syndrome cytokine profiles following treatment and relapse

Serum samples from thirty-eight patients were matched pre- and post-treatment, and analysed to determine which cytokines were significantly altered following therapy. Cytokines from 10 of the patients with relapsed disease were compared against their post-treatment levels. VEGF and IL-7 both displayed high levels at presentation,

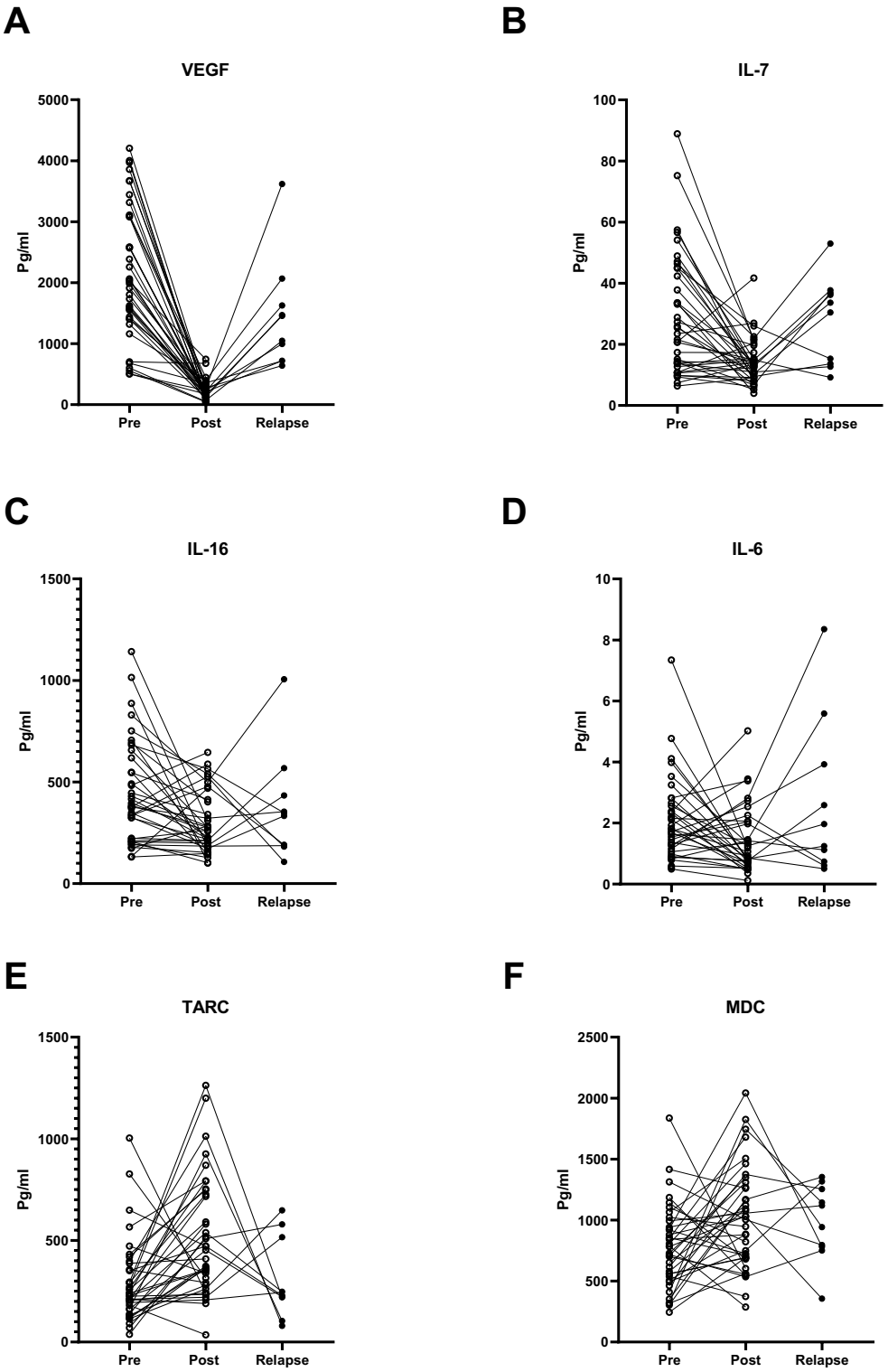
significant reductions following therapy, and significant increases in relapse. These two cytokines therefore appear to correspond with disease activity in a reliable manner. Levels of IL-16, Eotaxin-3 and IL-6 also significantly decreased following therapy, and although not statistically significant (possibly because of the small number of relapsed cases and broad confidence interval), additionally increased in relapsed disease (see figure 4.3). TARC, MDC and IL-17a levels all increase following therapy, and reduce in relapsed disease, although to a level higher than pre-treatment and again without statistical significance. Although levels of IP-10 and IL-13 did not alter with therapy, levels increased significantly in relapsed disease, and the potential significance of such will be discussed. Note that the LLOQ of IL-17a is 3.19pg/ml and 4.21pg/ml for IL-13 (as per kit certificates of analysis), and thus results may not be deemed quantifiable at lower levels. Differences in cytokine levels between groups in these two cytokines may therefore be due to assay imprecision rather than true reflections of disease activity.

Post treatment levels of cytokines in the patients who later relapsed were compared to those from patients who remained in remission (at the time of study) to ascertain whether the degree of cytokine suppression (or lack of) may influence the likelihood of relapse. Of the cytokines which significantly increased or decreased following treatment, IL-6 was the only post-treatment cytokine which differed significantly in relapsed cases (1.61pg/ml) to those with sustained remission (1.30pg/ml, p=0.004), however the difference is small, and with small patient numbers in the two groups should be interpreted with caution.

Table 4.5 Serum cytokine levels in pre-treated post-treated and relapsed POEMS

Cytokine	Pre-treatment (n=38) pg/ml Mean (CI)		Post-treatment (n=38) pg/ml Mean (CI)		P-value (vs pre)	Relapse (n=10) pg/ml Mean (CI)		P-value (vs post)
Decreased post-treatment								
VEGF	2158	(1797-2518)	229	(178-279)	**<0.0001**	1436	(795-2076)	**<0.005**
IL-7	29.3	(22.8-35.8)	14.0	(11.6-16.4)	**<0.0001**	27.87	(17.5-38.1)	**<0.05**
IL-16	447	(364-530)	294	(244-344)	**<0.005**	371	(183-558)	NS
Eotaxin-3	16.0	(12.2-19.8)	11.5	(9.32-13.7)	**<0.05**	17.9	(5.21-30.6)	NS
IL-6	2.12	(1.65-2.60)	1.08	(1.02-1.76)	**<0.05**	2.66	(0.813-4.51)	NS
Increased post-treatment								
TARC	290	(222-358)	513	(416-610)	**<0.0005**	309	(167-451)	NS
MDC	774	(663-886)	1012	(872-1153)	**<0.01**	966	(712-1220)	NS
IL-17a	1.38	(0.994-1.77)	2.67	(1.80-3.53)	**<0.005**	2.60	(-5.55-9.59)	NS
Increased on relapse only								
IP-10	99.3	(58.9-139)	96.9	(61.3-132)	NS	470	(299-622)	**0.019**
IL-13	0.794	(0.440-1.13)	0.794	(0.363-0.971)	NS	1.46	(0.778-2.15)	**0.039**
No change on treatment								
GM-CSF	0.18	(0.092-0.276)	0.221	(0.148-0.342)	NS	0.012	(-0.045-0.078)	NS
IL-12/p40	172	(133-210)	215	(171-259)	NS	154	(76.1-232)	NS
IL-15	3.43	(2.89-3.91)	3.71	(3.25-4.17)	NS	3.65	(2.91-4.39)	NS
IL-1alpha	1.68	(0.983-2.39)	1.63	(0.383-2.88)	NS	NA		NS
IL-5	0.343	(0.234-0.445)	0.513	(0.314-0.711)	NS	0.491	(-0.675-1.65)	NS
TNF-1b	0.126	(0.04-0.205)	0.263	(0.142-0.371)	NS	0.063	(-0.000-0.135)	NS
Eotaxin	244	(181-306)	232	(195-270)	NS	285	(198-372)	NS
IL-8	354	(160-548)	337	(244-430)	NS	204	(-155-565)	NS
MCP-1	326	(272-380)	329	(276-382)	NS	303	(178-431)	NS
MCP-4	176	(139-214)	167	(136-198)	NS	131	(83.5-179)	NS
MIP-1a	33.8	(23.6-43.5)	29.5	(22.2-36.8)	NS	18.9	(15.3-22.6)	NS
MIP-1b	160	(128-192)	169	(95.1-242)	NS	138	(82.0-194)	NS
INF-y	6.65	(4.41-8.89)	9.49	(5.74-12.2)	NS	6.57	(1.55-11.7)	NS
IL-10	0.683	(0.442-0.928)	0.732	(0.282-1.18)	NS	0.534	(0.025-0.971)	NS
IL-12p70	0.274	(0.204-0.342)	0.287	(0.207-0.352)	NS	0.283	(0.125-0.438)	NS
IL-1β	0.262	(0.120-0.417)	0.366	(0.021-0.702)	NS	0.242	(-0.115-0.597)	NS
IL-2	0.285	(0.154-0.419)	0.343	(0.210-0.396)	NS	0.81	(-0.012-1.67)	NS
IL-4	0.036	(0.022-0.041)	0.041	(0.01-0.705)	NS	0.021	(0.000-0.034)	NS
IL-8(b)	162	(34.7-291)	89.3	(-3.39-182)	NS	34.49	(7.91-61.0)	NS
TNF-α	3.36	(2.75-3.97)	4.06	(3.23-4.89)	NS	3.6	(2.41-4.85)	NS

Figure 4.4: Cytokine levels in pre-treatment POEMS cases compared to post treatment and relapsed disease



Serum VEGF (A), IL-7 (B), IL-16 (C) and IL-6 (D) demonstrating reduction following treatment, and increase in relapse. TARC (E) and MDC (F) displaying the inverse with increases post-treatment and reductions upon relapse

Changes in cytokine levels following treatment appear to result in (or co-exist with) reduction of POEMS symptoms, paraprotein levels, the difference in free light chains and improvement of ONLS. The overall reduction of cytokine levels does not particularly differ dependent on the treatment modality utilised, suggesting that improvement in POEMS syndrome is in part related to an overall reduction of cytokine levels, either as disease drivers or as markers of disease activity.

Table 4.6 Serum cytokine levels following different POEMS directed therapies.

	Radiotherapy	Lenalidomide	ASCT
Number (n=38)	5	18	15
Symptoms to diagnosis (m)	11	13.5	9
Total number symptoms	8	7	8
Pre	8	7	8
Post	4	3	3
Paraprotein pre (g/L)	2.6	3.2	3.8
Paraprotein post (g/L)	0.5	1.2	0.3
dFLC pre	21	25.9	18.4
dFLC post	2.2	5.82	8.31
ONLS pre	6	6.5	5
ONLS post	5	4	4
Progressed/died (%)	3 (60)	6 (33)	1 (6)
VEGF			
Pre	2667	1864	2339
Post	306	187	253
Delta	-2361	-1677	-2086
IL-7			
Pre	21.7	39.7	52.1
Post	12.7	15.1	12.5
Delta	-9	-24.6	-39.5
IL-16			
Pre	320	424	516
Post	258	253	353
Delta	-62	-171	-163
IL-6			
Pre	1.57	2.35	1.87
Post	2.97	1.32	1.02
Delta	1.4	-1.03	-0.85
Eotaxin-3			
Pre	15.2	15.3	17.0
Post	12.0	10.2	12.8
Delta	-3.2	-5.1	-4.2
IP-10			
Pre	112	124	64.5
Post	119	91.2	96.2
Delta	7	-32.8	31.7
MDC			
Pre	606	710	908
Post	1020	975	1056
Delta	414	265	148
TARC			
Pre	316	340	222
Post	442	468	588
Delta	126	128	355

Clinical features expressed as median values, paraprotein and cytokine levels as mean. dFLC= difference in light chains i.e. kappa and lambda on serum free light chain analysis (mg/L).

4.4 Discussion

This study measures more cytokines than any previous reports, in the largest number of POEMS syndrome cases. Primarily, this study demonstrates clear evidence of cytokines as biomarkers of POEMS syndrome. A number of previously unreported relevant cytokines have been discovered which are specific to POEMS syndrome, and may further develop our knowledge of the underlying pathological mechanisms of disease.

4.4.1 Key cytokines identified in POEMS syndrome sera

Several cytokines were raised in active, pre-treated POEMS syndrome compared to matched disease groups and/ or healthy controls including VEGF, IL-7, IL-16, IL-6, IL-4, MIP-1α, Eotaxin-3, IL-15, IL-5, MCP-1, TNF-α, and MIP-1β. This broad range of cytokines provides further evidence of the 'cytokine storm' which appears to underpin POEMS pathogenesis. Cytokine storms, that is the uncontrolled release of proinflammatory cytokines, are known to occur in response to a wide range of infectious and non-infectious diseases, and are currently of significant research interest due to their occurrence in COVID-19 infection. [217] The cytokine storm occurs as a consequence of a number of haematological conditions such as graft vs host disease, following novel CAR-T therapies and notably in Castleman's syndrome, the latter sharing several features of POEMS syndrome. Although the complex nature of this immune system overdrive is poorly understood in POEMS syndrome, it appears that the clonal plasma cell expansion directly and indirectly produces of a number of key cytokines, which attract immune cells and activate further signalling pathways, leading to an immunological cascade, amplifying and broadening the proinflammatory response, resulting in positive

feedback and further inflammation. This systemic activation is likely to contribute towards the several seemingly unrelated comorbidities of POEMS syndrome. The V-PLEX 30 cytokine assay used for this study appeared appropriate to study the broad range of inflammatory molecules involved in cytokine storms. This kit consists of interleukins, interferons, chemokines, colony stimulating factors (CSFs) and tumour necrosis factor. Broadly, interleukins are responsible for growth and differentiation of leukocytes, interferons regulate innate immunity to viruses and other microbial pathogens, chemokines control migration of immune cells to sites of inflammation, and CSF's stimulate haematopoietic progenitor cells proliferation. Tumour necrosis factor is perhaps the most studied proinflammatory cytokine, and is thought to play a predominant role in the cytokine storm. TNF exerts its pleiotropic effects to activate signalling pathways that regulate cell survival, proliferation or death.[218] Although the concept of a cytokine storm in POEMS syndrome appears a fundamental aspect of POEMS syndrome pathology and symptomatology, attempting to unravel the individual role of key markers of inflammation may allude to a better understanding of the critical steps leading up to this, and potentially identify important biological processes or therapeutic targets.

Levels of VEGF, IL-7, IL-16 and IL-6 were significantly raised in POEMS syndrome compared to diseased matched and healthy controls, and reduced significantly following therapy. Eotaxin-3 displayed the same features, although levels were comparable in pre-treated CIDP cases. These cytokines subsequently increased in relapsed disease. The potential pathological relevance of such cytokines shall be discussed in further detail.

4.4.1.1 Interleukin 7

Of all the 30 cytokines analysed, Interleukin 7 (IL-7) appears of considerable relevance. IL-7 is considered a 'master controller' of both the adaptive and innate immune systems. IL-7 receptors (IL-7R) are predominantly expressed by lymphocytes, notably T cells, NK cells and B-cells, but are also found on various cellular components of the innate immune system such as macrophages, dendritic cells and lymphoid tissue inducer cells.[219] This broad expression profile provides evidence towards the pleotropic effects of IL-7 on other immune cells. IL-7 is secreted largely by specialised stromal cells (mostly of the bone marrow, spleen and thymus) and vascular endothelium in sites of lymphopoiesis (the process of lymphocyte development from progenitor cells).[220] For B cells, this occurs in the bone marrow and results in the production of differentiated T, B, natural killer (NK) and plasma cells. IL-7 is thought to be responsible for B cell maturation and proliferation, demonstrated by upregulation of IL-7R's on Pre-pro B cells and their progeny, Pro-B and Pre-B2 cells.[221] In addition, IL-7 has been demonstrated to regulate immunoglobulin gene rearrangement, and thus may contribute towards the neoplastic lambda light chain restricted immunotype of plasma cells.[222–224] For T cells, lymphopoiesis occurs in the thymus, resulting in several forms of thymocytes including T-helper cells, cytotoxic-T cells, T-memory and T-suppressor cells.[225] B and T cell development appears to be dependent on IL-7, whereby IL-7$^{-/-}$ and IL-7R$^{-/-}$ mice exhibit a severe reduction in both B and T cell numbers. [226] The development of lymph nodes relies on cross talk between haematopoietic CD4^{+} IL-7 receptor positive lymphoid tissue inducer cells and ICAM-1 positive mesenchymal organiser cells. It is conceivable that IL-7 stimulates lymph node hyperplasia and maturation of B cell clones within secondary lymphoid tissue in POEMS syndrome.[227]

Overexpression of IL-7 in transgenic mice may provide a model for the pathogenic effects of IL-7. The authors of basic discovery studies of the production of IL-7Tg mice 'opened a Pandora's box of phenomena' including a massive increase in bone marrow B cell lymphopoiesis,[228] increase in T cell numbers,[229] development of bi-potent B/myeloid lymphomas,[230] development of Peyer's patches (aggregated lymphoid nodules), lymph nodes [231] and bone lesions.[232] An expansion of the bone marrow cavity occurred, and cortical bone demonstrated focal osteolysis. The number of pro-B cells, macrophages and plasma cells were increased. The authors suggested that prolonged stimulation of B cell precursors in the bone marrow by IL-7 could lead to neoplasms. Although *in vivo* animal models do not always reflect similar pathogenesis in humas, the features described are reminiscent of POEMS syndrome, notably the haematological malignancy, bone lesions and lymph node hyperplasia.

Although the clonal plasma cell population in POEMS syndrome is relatively small, this is a key aspect of disease. Both monoclonal and surrounding polyclonal bone marrow plasma cells are thought to contribute to the production of VEGF and possibly other cytokines.[109] At three times the level of that in related disease and healthy controls, it is plausible IL-7 contributes towards the proliferation and maturation of the pathogenic monoclonal and polyclonal plasma cells. If such plasma cells themselves secrete IL-7, this will lead to a positive feedback, accentuating the response. IL-7 levels also correlated with VEGF pre- and post-treatment, and in relapsed disease. It may be that IL-7 stimulates neoplastic and non-neoplastic plasma cell proliferation, which in turn secrete VEGF. Levels of IL-7 did not correlate with paraprotein level, SFLC ratio, neoplastic plasma cell proportion in the bone marrow or difference in free light chains

at presentation, however this may require further scientific exploration now IL-7 has been identified as relevant. Levels of IL-7 decreased following therapy, in keeping with a significant reduction in the aforementioned haematological parameters (table 4.5). Further work may be conducted to investigate whether persistently raised levels of IL-7 following treatment are associated with lack of haematological response and subsequent POEMS relapse.

The finding of IL-7 as a potentially pathogenic cytokine in POEMS syndrome raises further questions regarding the influence of other proinflammatory markers on B cell proliferation and survival in POEMS syndrome, which could contribute as drivers of the clonal plasma cell disorder. For example, IL-7 is known to induce the B cell regulatory proteins CD70 and B cell activation factor (BAFF) in resting T cells which leads to B cell proliferation.[233] B-lymphocyte stimulator (BlyS), A proliferation-inducing ligand (APRIL), BAFF and their receptors 'transmembrane activator and CAML interactor' (also called TACI), B cell maturation antigen (BCMA) and BAFF receptor are important for B cell proliferation and have been demonstrated as potential drivers for multiple myeloma,[234] and thus may be relevant areas for exploration in POEMS syndrome. In addition, bispecific antibody constructs, antibody drug conjugates and chimeric antigen receptor (CAR)-modified T-cell therapies are being developed to target BCMA in myeloma. [235]We are currently planning on carrying out this work. Assays for such B-cell markers are commercially available on ELISA platforms, or MSD. The advantage of an MSD panel is the ability to multiplex and create a custom designed B -cell immunoassay at higher sensitivities than that of an ELISA, but at significantly greater cost.

4.4.1.2 Interleukin 16

Interleukin 16 (IL-16) functions as a regulator of T cell growth and recruiter of CD4+ve hematopoietic cells during inflammatory responses. IL-16 is predominantly secreted by activated CD8+ve T lymphocytes, but also by a range of other haematopoietic cells.[236] The primary receptor for IL-16 is CD4R, which is expressed on several haemopoietic cells, but also on neuronal cells.[237] The main function of IL-16 is therefore as a chemoattractant of CD4+ve T helper 1 and 2 lymphocytes, although other cell types such as eosinophils, dendritic cells, monocytes and B cells which express CD4 have been demonstrated to be responsive to IL-16 stimulation. Th1 cells stimulate cell mediated responses (mostly via cytotoxic T cells and macrophages) compared to Th2 cells which primarily assist stimulating B cells to produce antibodies. Increased IL-16 levels also activate the expression and production of proinflammatory cytokines including IL-6, IL-15, IL-1β and TNF-α from monocytes.[238] More recently, IL-16 has been correlated with the onset and development of various haematopoietic cancers. Koike et al have demonstrated that IL-16 is highly expressed in the bone marrow stroma and found in the serum of patients with multiple myeloma, levels of which correlate with disease severity.[239,240] IL-16 has been shown to support the proliferation of clonal plasma cells, possibly through involvement of transcription factors FOS and JUN.[241] CD8 cell activation leading to IL-16 release, and subsequent activation and exhaustion/death of CD4 cells has been demonstrated to occur in multiple myeloma, and function as a biomarker of disease activity. T cells play a crucial role in anti-tumour immune responses and efficacy of immunotherapies, however their role in POEMS syndrome is poorly understood and requires further research.[242]

It is unknown whether IL-16 production is directly from the abnormal plasma cells in POEMS syndrome, or is secreted by other haematopoietic cells in response to activation/ stimulation from other cytokines. Now that a number of relevant cytokines have been identified through this study, further research investigating the source of such cytokines would be important to our understanding of disease. This could be performed by culturing myeloma bone marrow cells and measuring cytokine levels in the supernatant, or through Realtime-PCR to measure RNA. Regardless of the source, it is likely IL-16 provides additional support to the aberrant plasma cell clone of POEMS syndrome, possibly in combination with IL-7. In addition, the secondary effect of CD4+ve cell activation and subsequent exhaustion may result in a degree of resistance to immunotherapies.

Levels of IL-16 in this study were only raised in POEMS syndrome and were not increased in the myeloma cohort, with levels comparable to healthy controls. Looking into this data further, most studies have stratified myeloma cases by severity scores such as the Durie-Salmon staging system based upon haemoglobin, calcium, M-protein and light chain values and number of bone lesions.[240] In a study by Alexandrakis et al for example, levels of IL-16 in stage I myeloma was 253pg/ml (144-418) and 270pg/ml (118-562) in stage II compared to 405pg/ml (161-1,259) in stage III.[240] It may therefore be conceivable that the IL-16 levels in myeloma cases in this cohort (196pg/ml, 95% CI 156-236)) were in less advanced stages to that of other studies, explaining the discrepancy.

IL-16 has a potential additional role in causing neuropathy in POEMS syndrome. IL-16 has been implicated in Experimental autoimmune neuritis (EAN), a well-recognised autoantigen-specific T cell mediated animal model of human demyelinating inflammatory diseases of the PNS, which may bear relevance in POEMS syndrome. The initiation of EAN is caused by autoreactive T cells recognising peripheral nerve autoantigens on antigen presenting cells such as monocytes or Schwann cells.[243] Following activation, these T cells attach to the endothelium in the PNS, penetrate the blood nerve barrier (BNB) and generate an immune reaction that orchestrates the invasion of further autoreactive T cells and macrophages. Activated macrophages cause demyelination by phagocytosis or via secreted inflammatory mediators. [244] Studies have demonstrated evidence of IL-16 expression in sciatic nerves, dorsal/ventral roots and spinal cords of EAN rats using immunohistochemistry, and demonstrated correlation with clinical severity and demyelination.[245] The major IL-16+ve cells were infiltrated macrophages and T cells. The percentage of IL-16+ve T cells to total T cells differed at different time points in EAN sciatic nerves indicating that alteration of IL-16 production was induced in EAN. It is therefore thought that IL-16 leads to T cell and macrophage migration and proliferation, and induction of proinflammatory cytokines from monocytes like TNF-α, IL-6 and IL-15.[238,246] This work could be replicated using sural nerve samples from POEMS and CIDP cases, staining for IL-16, comparing levels and correlation with disease activity. Other studies using T cell depletion have demonstrated CD4, but not CD8 T cells as important mediators of disease in EAN, responsible for cytokine production, regulation of macrophages and mediating axonal damage. This is in keeping with the above proposed mechanism of IL-16, activating CD4 cell populations.[247] In EAN, macrophages are considered to be the predominant effector destroying myelin. In POEMS syndrome, IL-16 levels were significantly higher than any

other disease cohort or healthy control. High serum IL-16 may therefore mediate CD4 T cell mediated macrophage activation and penetration of the BNB in POEMS syndrome. Levels of IL-16 in CIDP are similar to healthy controls. Although CIDP causes peripheral nerve demyelination (similar to POEMS syndrome), the presumed pathological mechanism and pattern of demyelination differs between the two conditions. CIDP is typified by more focal proximal and distal demyelination, which is possibly antibody mediated (explaining the normal IL-16 levels) compared to POEMS syndrome which is a cytokine driven process.

4.4.1.3 Interleukin 6

Interleukin 6 (IL-6) has been previously demonstrated to be a marker of disease activity in POEMS syndrome, further supported by this study. The pleotropic effects of IL-6 on several body systems (figure 4.1) holds a degree of face validity as being a potentially pathogenic cytokine in POEMS syndrome. IL-6 plays a central role as a differentiation and growth factor for haematopoietic precursors, B cells, T cells, keratinocytes, neuronal cells, osteoclasts and endothelial cells.[248] Secreted by immunocompetent cells (T, B cells and macrophages), hematopoietic cells, endothelial, epithelial cells and osteoblasts, IL-6 has been demonstrated as being involved in a number of B cell mediated haematological diseases, including Castleman's disease, plasmacytomas, myeloma and POEMS syndrome. [249] In CD, the main production of IL-6 is from germinal centres of hyperplastic lymph nodes.[250] It may be that IL-6 production is additionally from lymph nodes in POEMS syndrome. Castleman disease lymph node histology has been detected in up to 73% of POEMS cases in the cohort from the United States.[9] In our cohort of 100 cases, 42 had enlarged lymph nodes on imaging, however this may be

under reported because of the nature of retrospective data collection. Eighteen had lymph node biopsies, in which the majority (14) demonstrated CD histology. To enable a better understanding of the lymph nodes in POEMS and their possible contribution to disease, more detailed study of the frequency of lymph node hyperplasia in POEMS, and the histology of enlarged and non-enlarged lymph nodes would be required, in addition to studying the cytokines expressed from such tissues. Due to the likely unreliable/inaccurate rate of reporting of CD in our POEMS cohort, we were unable to determine whether cases with higher levels of IL-6 had CD histology or not.

The bone marrow is also an additional likely source of IL-6. As seen in POEMS, myelomatous plasma cells secrete VEGF, including other cytokines such as IL-1β, TNF-α and TNF- β.[109,251] These cytokines then stimulate bone marrow stromal IL-6 production, which binds to IL-6Rs on plasma cells leading to proliferation and promoting cell survival, with levels correlating with clinical outcome.[252] IL-6 is additionally known to affect bone turnover. Osteoblasts express IL-6Rs, and IL-6 has been shown to result in increased bone formation, whilst additionally stimulating osteoclast differentiation and bone resorption.[253] The interaction between tumour cells and bone marrow stromal cells appears to play a critical role in the production of bone lesions in plasma cell malignancies. It is therefore likely that bone marrow derived IL-6 in POEMS syndrome functions to propagate the monoclonal plasma cell clone, stimulating local and systemic VEGF production causing a number of downstream effects. Increasing bone turnover is likely to contribute towards the characteristic mixed sclerotic and lytic bone lesions, and possibly the development of further plasmacytomas due to local effects on propagating aberrant plasma cell clones.

IL-6 stimulates tumour microenvironment angiogenesis and has a direct effect upon endothelial cell proliferation and migration, with similar potency to VEGF. In contrast to VEGF, IL-6 stimulated vessels have defective pericyte coverage in animal studies using the aortic ring as a commonly researched model of angiogenesis.[254] Pericytes maintain homeostatic function of the endothelium and are important in the formation and regulation of the blood brain and blood nerve barriers.[255] Systemic hypercytokineaemia, particularly with high serum concentrations of VEGF and IL-6 are likely to contribute towards profound vascular leak resulting in peripheral oedema, ascites, pleural effusions and papilloedema. Pericyte dysfunction leads to breakdown of the blood nerve barrier, which either in isolation leads to nerve damage through excessive oedema and swelling, or likely allows a 'toxic' neuropathy to develop by allowing neuropathic cytokines and other molecules to come into contact with the nerve and its microenvironment. Although the histopathological changes associated with the blood nerve barrier disruption in POEMS syndrome have not been studied, MRI imaging and sural nerve biopsies in POEMS syndrome display oedema, neovascularisation and endothelial hyperplasia likely reflecting the effects of both IL-6 and VEGF on the BNB.[1,16,107] Neuropathy in POEMS syndrome is more severe than that of POEMS with CD, which is more severe than neuropathy in CD alone. The pathological mechanism underlying this phenomenon is not known. Although IL-6 production is thought to contribute towards the features of CD (systemic inflammation, cytopaenias and multi-organ dysfunction), several studies have not been able to demonstrate high levels of IL-6 in the serum of such patients.[256,257] The elevated IL-6 (and VEGF) which has been consistently demonstrated in the serum of POEMS syndrome cases is likely to lead to more severe disruption to the endothelium with several effects such as thrombosis, vascular leak, papilloedema and most importantly, neuropathy. Studying the cytokine

profiles of CD, POEMS-CD and POEMS alone may provide important distinctions which reflect the varied clinical presentations, and possibly provide evidence towards the above hypothesis.

4.4.1.4 TNF alpha

Tumour necrosis factor alpha (TNF-α) was raised in the sera of POEMS syndrome cases in this study, but also in CIDP and myeloma which is expected. As mentioned earlier, TNF-α has been demonstrated to be toxic to myelin, Schwann and endothelial cells, with levels raised in the sera and expressed in peripheral nerves of GBS, paraproteinaemic neuropathies and CIDP.[258] Similar to IL-6, TNF-α is a survival factor for myeloma cell lines, and is raised in the sera of patients on presentation.[259,260]

4.4.1.5 Interleukin 4, 5 and CC chemokines

In this study, although under the quantitative limit of the assay, levels of Interleukin 4 (IL-4) were significantly raised in POEMS syndrome compared to other diseases. IL-4 activates endothelial cells which produce a variety of chemokines regulating eosinophil chemotaxis including CCL5 (aka RANTES), eotaxin, eotaxin-2, eotaxin-3, MCP-1 and MCP-4. Of this list, eotxin-3, and MCP-1 were additionally increased in POEMS syndrome. Monocyte chemotactic proteins (MCPs) and the eotaxins constitute a subfamily of CC chemokines that share approximately 65% amino acid identity[261] These chemokines target the CCR3 receptors primarily on eosinophils and basophils leading to activation.[262] IL-5, also raised in POEMS syndrome, provides a similar functional role in eosinophil proliferation.[263] Eosinophils perform numerous tasks. They are primarily

involved in innate immune defence against parasites and in response to allergens. Recently, however, it has become clear that eosinophils also play a crucial role in the generation and maintenance of adaptive immune responses.[264] Eosinophils are a major source for the plasma cell survival factor APRIL and IL-6 which would further support both the aberrant and polyclonal plasma cells in POEMS syndrome.[265] Eosinophil activation and trafficking has not been previously considered in POEMS syndrome pathogenesis, and are not routinely tested. In addition, eosinophils are not found in peripheral nerve samples of POEMS syndrome cases, suggesting they do not play a role in peripheral nerve pathogenesis. This pathway may have been overlooked in this case if a solitary cytokine were identified. However, the raised IL-4, IL-5, MCP-1 and eotaxin-3 in disease, and correlation of eotaxin-3 with MCP-1 firstly demonstrates that a chemokine storm is also a feature of POEMS syndrome, but downstream this may lead to Th2 pathway eosinophil and basophil activation. Whether eosinophil and basophil activation have a pathogenic role in POEMS syndrome is unknown. Such markers may otherwise simply reflect an immune hyperactivation, chemokine storm and chronic inflammatory state. Flow cytometry of POEMS syndrome sera could be performed to quantitate eosinophil expression compared to that of CIDP, myeloma and healthy controls. Commercial kits are also available allowing for eosinophil purification from peripheral blood, which can be used for cell culture, activation and measurement of secreted products. [266]

The macrophage inflammatory proteins-1a and b (MIP-1a, MIP-1b) are additional CC chemokines raised in POEMS syndrome. MIP-1a and 1b are secreted primarily by macrophages, but also by a number of activated mature haematopoietic cells. MIP-1a attracts CD8 T lymphocytes and B cells, whereas MIP-1b attracts naive helper T cells.

Both induce the secretion of TNF-α, IL-1α and IL-6 from macrophages.[267] MIP-1a is secreted directly by myeloma cells, enhances plasma cell proliferation and survival, and correlates with disease stage, and thus may have similar properties in POEMS syndrome.[267] Within the bone marrow, MIP-1 has been demonstrated to increase adhesive interactions between myeloma cells and stromal cells which results in increased production of IL-6 and VEGF, leading to further plasma cell growth, local and systemic angiogenesis and bone destruction.[268]

Bone lesions are very common in both myeloma and POEMS syndrome, and appear to differ from lesions of other tumours which involve the bone. This may be inherent to the cytokine milieu of both conditions which appears to be at least in part driven by the bone marrow haematopoietic cells. MIP-1a acts as a chemotactic factor for osteoclast precursors leading to lytic bone lesions which are characteristically seen in both myeloma and POEMS syndrome, although POEMS more often exhibiting a mixed sclerotic/lytic picture. IL-7 is also known to inhibit osteoblast activity which will further potentiate bone lesions.[269] The influence of cytokines and chemokines on osteoblast and clast activity appear to influence the development of bone lesions in POEMS syndrome. Although not all bone lesions are biopsied, we know from experience that a proportion demonstrate malignant clonal plasma cell infiltration (which often display activity on FDG-PET), however some do not. It is unclear whether those that do not are remnants of previous active disease, or if they have developed independent of clonal plasma cell proliferation and solely in response to cytokine activated bone turnover. Studying serial imaging (particularly FDG-PET) would enable better understanding of the natural history (and possibly the pathogenesis) of bone lesions in POEMS syndrome.

Chemokines appear to have a potentially important neuropathological role in POEMS syndrome and related inflammatory neuropathies. In EAN mice, sequential expression of chemokines was found using homogenised sciatic nerves. Increased expression of MIP-1a and MIP-1b preceded disease, with IP-10, CCL5 and MCP-1 concomitant with maximal clinical disease.[270] It was therefore suggested that MIP recruits inflammatory Th1 lymphocytes and monocytes into the neural parenchyma, and MCP-1 and RANTES amplify the immune response. MIP-1a and MIP-1b has also been demonstrated to be raised in the sera of CIDP patients, supported by this study, with levels similarly raised in POEMS and CIDP, and not in myeloma or healthy controls.[271]

TARC and MDC are also CC family chemokines which are highly expressed in the thymus and secondary lymphoid tissue (predominantly by macrophages) and stimulated by IL-4 and TNF-α. Both chemokines bind to the receptor CCR4, activating Th2 responses to guide B-cell responses.[272] Both cytokines are reduced in POEMS syndrome, and increase following treatment. The significance of such findings is unknown, and appears to contrast that of other chemokines and their presumed influence on Th2 pathways. This may be a secondary immunosuppressive effect, whereby B cell activation and systemic hyperinflammation results in a compensatory downregulation of non-bone marrow derived immune activity.

IP-10 is a chemokine of interest in the field of inflammatory peripheral nerve pathogenesis, and was at a significantly lower concentration in POEMS cases compared to CIDP and myeloma, and more comparable to that of healthy controls. IP-10 is thought to be released from the blood nerve barrier in immune mediated neuropathies.[273] TNF-α also induces IP-10, and since levels of TNF-α were raised in POEMS, the lack of IP-10

appears to be of significance, underpinning the different pathologies of the two conditions. IP-10 and/or its receptor has been detected in human and EAN mice peripheral nerve histopathological samples, and in CSF of patients with CIDP, but not compared in the serum.[270,271,274] Expression is localised to perineurial endothelial cells in EAN models, suggesting its role in transendothelial T lymphocyte trafficking.[270] The significance of raised serum IP-10 in both CIDP and myeloma is unknown, as is the lack of IP-10 in POEMS syndrome. However, peripheral nerve biopsies in POEMS syndrome characteristically display very few lymphocytes in the endo, epi or perineurium, with marked oedema and epineural neovascularisation as typical features, compared to CIDP which has more marked T lymphocyte inflammation.[17,18,84] The lack of IP-10 in POEMS syndrome may account for this difference, whereby neuronal damage is more related to a toxic inflammation of nerves secondary to damage to the blood nerve barrier and alterations in the peripheral nerve microenvironment, with exposure to high levels of potentially neurotoxic inflammatory cytokines, compared to CIDP which is more typically autoimmune and secondary to lymphocytic inflammation and macrophage activation. More unexpectedly, levels of IP-10 do not alter upon treatment but significantly rose to levels higher than that of CIDP and myeloma upon relapse. The significance of this is unknown, and in 10 cases only, again, should be interpreted with caution.

4.4.2 Comparing data with the existing literature

Comparing the results of this study to those already published, levels of VEGF, TNF-α and IL-6 have been found to be consistently raised in POEMS syndrome compared to other diseases. IL-1β was not raised in this study which contrasts other reports (table

4.1). This may be assay dependent, as we have subsequently used this assay in a separate unrelated study, and found similar results (low levels in cases where IL-1β would be expected to be high).[275] Cytokine levels can be affected by sample preparation and storage, but the methods employed in the other study were more controlled than this (i.e. with samples being drawn and immediately frozen and delivered to the laboratory) and produced similar results, refuting this hypothesis. [275] Kuwabara et al have also performed multiplex cytokine analysis on POEMS sera, and found 12 markers to be raised in POEMS. They identified IL-12p70 as being a novel biomarker of disease activity due to levels being markedly raised on presentation (78.7 pg/ml) and significantly reducing on treatment (30.1 pg/ml).[192] Other than defining that IL-12p70 plays a role in acquired immunity, involved in the Th1 response, little interpretation is made with regards to the role of IL-12 in POEMS pathogenesis, but their data does implicate the cytokine as being relevant. We measured both IL-12p70, and the individual 40-kDa subunit IL-12p40. Neither were raised in active POEMS syndrome, responded to treatment or increased upon relapsed disease. Levels of IL-12p70 in this study were measured under 0.5 pg/ml for POEMS and all other conditions, compared to that of the Kuwabara study using a different assay kit (Bio-Plex human 27-Plex panel, Bio-Rad, Hercules, CA). Looking at our data, the standard curve for IL-12p70 demonstrated a broad dynamic range from 0.135 pg/ml to 552 pg/ml, with coefficient of variance (CV)% of 0.2-5.6% on duplicate calibrator values. The MSD V-PLEX 30 kit however does not contain internal controls which would be helpful to assess whether the assay can accurately measure a known concentration of analyte. This could also determine if matrix effects exist which may inhibit the endogenous measurement of IL-12p70 in the serum samples compared to that of the calibrator diluent. Kuwabara measured serum samples and reported no matrix effects. The MSD VPLEX-kit validation

data displays the typical cytokine concentrations tested on 27 human normal control samples, and for IL-12p70 displays a median of 0.29 pg/ml, with a range of 0.26-0.38 pg/ml, and only 11% of samples were found to have concentrations over the LLOD. When comparing this to the levels of IL-12p70 measured in the Kuwabara study (10.7 pg/ml), this demonstrates the two assays are non-comparable. The very low (and often undetectable) concentration of IL-12p70 measured in the healthy controls of the MSD kit may be true and accurate, however combined with the similar results in disease cases, particularly POEMS, would suggest the MSD kit is not reliable in measuring this cytokine accurately. It would therefore be advisable to repeat testing for Il-12p70 using the same Bio-Rad kit with the same samples to determine whether the results could be replicated.

Our study has discovered a number of cytokines not previously identified as being raised in POEMS syndrome, which may have a role to play in the multi system manifestations of disease. As mentioned, the cytokines VEGF, IL-7, IL-6 and IL-16 appear to be most significantly raised in POEMS syndrome, reducing upon treatment and spiking once again on relapse. The physiological relevance of such cytokines, and their potential interplay is hypothesised in section 4.4.1, and illustrated in figure 4.5, but requires further exploration. These cytokines in combination might in future be considered as biomarkers both for diagnosing POEMS syndrome and measuring underlying disease activity. Despite the four cytokines in combination predicting a diagnosis of POEMS syndrome more accurately than VEGF alone in this cohort, the area under the curve (reflecting the sensitivity and specificity) of VEGF reflects the diagnostic capability of this test independently, and thus probably negates the need for

further specialist testing to aid in the diagnosis. However VEGF has also been demonstrated to be elevated in cases of iron-deficient anaemia and chronic hypoxic states.[2] In such circumstances the combined biomarker assay is likely to be diagnostically useful with better assay specificity.

4.4.3 Limitations

We recognise bias in this retrospective study, and acknowledge prospective testing of a validation cohort is of importance. The POEMS cohort in this study were selected based on fitting the diagnostic criterion for POEMS syndrome and thus all cases selected for this study therefore had a raised VEGF level by definition. Our cohort data of 100 cases demonstrates 94% of cases were found to have raised VEGF on presentation (table 2.1), and it is possible that those recorded as having normal levels may have been pre-treated with steroids which can lower VEGF levels. It will be of interest to study cytokine levels in cases which have low or borderline low VEGF levels to see whether other markers of disease activity remain high. Interestingly, levels of the cytokines deemed relevant in this study did not correlate with clinical markers such as ONLS score, or haematological indices such as percentage infiltration with plasma cells on bone marrow or difference in the SFLC, or prediction of death or progression in POEMS syndrome. This finding is consistent with what is already known about VEGF, where clinical findings do not always correlate in a 'dose dependent' fashion with serum concentrations. What appears to be more relevant in POEMS syndrome, particularly with neuropathy is the passage of time, which might be considered as 'exposure time', that is of organ systems to high circulating cytokine levels. Patients in whom the diagnosis of POEMS was made in less than six months from symptom onset had

significantly lower ONLS scores at four, compared to those diagnosed after six months at six ($p<0.05$) suggesting that delay to diagnosis, and thus possibly exposure time to pathogenic cytokines influences neuropathy severity and overall long-term disability more than cytokine concentrations alone.

4.4.4 How do these data advance our knowledge of POEMS syndrome pathogenesis?

This study has improved our knowledge of at least some of the immune mediators and pathways that appear inherent to POEMS pathogenesis. This has been summarised in figure 4.5, and can be compared to that of figure 1.3 which was compiled prior to this thesis being undertaken, demonstrating the development of our understanding. Although a number of cytokines have been identified at high levels in the sera of POEMS patients, further work will be required to unravel their pathogenic role in POEMS syndrome and particularly in neuropathy. However, interpreting this data, a number of hypotheses can be proposed regarding the pathogenesis of POEMS syndrome:

- Firstly, IL-7 appears to be produced by the vascular endothelium and stroma of bone marrow leading to lymphopoiesis of a number of immune cells including T and B lymphocytes, macrophages and dendritic cells, present in the bone marrow circulation.
- IL-7 restricts immunoglobulin rearrangement from B cells promoting the development of lambda light chain restricted plasma cell clones, likely in the presence of other co-stimulatory B cell regulatory proteins such as BAFF, APRIL and B cell maturation antigen.

- Circulating IL-7 also stimulates lymph node hyperplasia and secondary lymphopoiesis of plasma cell maturation and development.
- The bone marrow monoclonal plasma cells and germinal centres in hyperplastic lymph nodes produce IL-6. In the bone marrow, IL-6 binds to IL-6Rs on the neoplastic plasma cells leading to monoclonal and surrounding polyclonal plasma cell proliferation.
- IL-6 induces the expression of VEGF into the systemic circulation at high concentrations.
- IL-16 is known to be expressed by the bone marrow stroma and by circulating CD8+ve T lymphocytes in response to cytokine activation. IL-16 functions as a chemoattractant of CD4+ve T helper 1 and 2 lymphocytes, which activate cytotoxic T cells and macrophages in cell mediated immunity, whilst also supporting the proliferation of clonal plasma cells in the bone marrow and promoting antibody production from B cells.
- Endothelial cells and macrophages are likely stimulated by circulating IL-7, IL-6, IL-16, IL-4 and IL-5 produce a chemokine storm in the blood serum, with increased expression of eotaxin-3, MCP-1, MIP-1a and MIP-1b. Such chemokines activate eosinophils and attract B cells and CD8+ve T lymphocytes which produce IL-16.
- MIP-1 increases adhesive interactions between plasma cells and stromal cells in the bone marrow which results in further IL-6, IL-7, and VEGF production.
- Circulating cytokines have a number of multi-system effects. IL-6 binds to IL-6Rs on osteoblasts stimulating bone formation whilst additionally accelerating osteoclast formation.

- MIP1a acts as a chemotactic factor for osteoclast precursors promoting bone lysis. Such processes lead to disorganised bone remodelling and bone lesions. These cytokines additionally promote B cell development and therefore propagate plasma cell clones within bone lesions causing plasmacytomas.
- VEGF and IL-6 stimulate angiogenesis through pericyte dysfunction at multiple organ sites including the liver, spleen, circulatory system, retinas, blood-brain and blood-nerve barrier. Angiogenesis and leakage of the hypercytokinaemic systemic circulation into organs leads to oedema, inflammation and sclerosis which disrupts venous return leading to organomegaly, in combination with peripheral oedema.
- VEGF and IL-6 affect haemostatic properties of endothelial cells by inducing von Willebrand factor and thrombomodulin, both of which aggregate platelets and modify fibrinolysis due to the stimulation of tissue-plasminogen activator resulting in arterial and venous thrombosis.[227]
- Neuropathy in POEMS syndrome occurs primarily due to IL-6 and VEGF mediated activation of endothelial cells within the endoneurium leading to microvascular angiogenesis, resulting in a secondary ischemic microangiopathy and blood nerve barrier hyperpermeability. This exposes the previously immune privileged peripheral nerves to the systemic circulation.
- IL-16 activated T cells recognise peripheral nerve antigens, generating an immune reaction activating macrophages and stimulating further production of cytokines, such as TNF-α which is directly toxic to myelin. The disruption of vascular and metabolic dynamics in the nerve leads to a perpetuating hypoxic state, where secondary increases in local VEGF occurs due to induced HIF-1a

expression, fuelling a positive destructive feedback whereby damage progresses relentlessly.

Results of this study demonstrate that a complex network of proinflammatory markers, cytokines and chemokines underpin POEMS syndrome pathogenesis. Such findings likely explain why blocking VEGF alone does not lead to disease suppression in POEMS syndrome. As detailed above, a number of the cytokines described are involved in VEGF pathways and thus may have been inhibited through VEGF blockade, however a significant proportion also appear independent, such as IL-7, and therefore a number of immunological pathways would continue to be active during anti-VEGF blockade alone. Lessons learnt from VEGF-blockade trials, combined with those data from Kuwabara and our cytokine study continue to support the notion that POEMS therapy should be directed towards the underlying plasma cell clone, which subsequently appears to 'turn down' the complex immune response either directly or indirectly, leading to suppression of the cytokine storm and disease remission. Unlike Castleman's disease, in which IL-6 blockade appears to result in significant improvements (and additionally supports the role of IL-6 secreting lymph nodes as the major disease-driver), it is unlikely that individual cytokine blockage in POEMS syndrome would be of clinical utility, based upon what is now understood regarding pathogenesis.

Although the identification of circulating cytokines in POEMS syndrome provide an indication of the possible neuropathological mechanism, further work needs to be done. It remains unknown whether several of the cytokines identified in this study are definitely produced by the malignant bone marrow plasma cells and/or the related

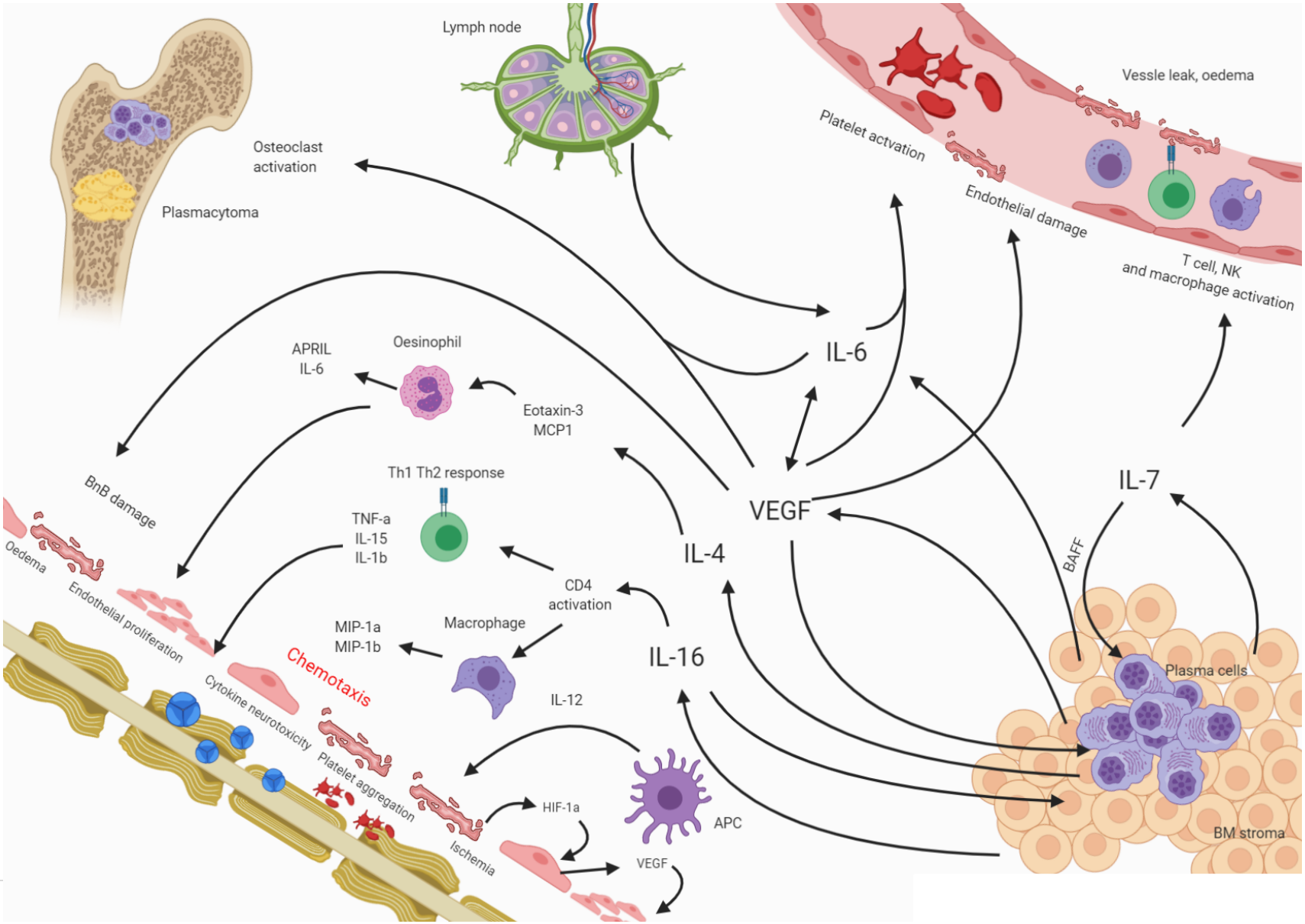
stroma, as has been postulated. Wang et al have demonstrated that polyclonal plasma cells are the major source of VEGF in POEMS syndrome. This was the first study to confirm a link between plasma cells and the production of VEGF in POEMS syndrome.[109] We are in the process of making plans to isolate bone marrow plasma cells in POEMS syndrome patients and test expression of the cytokines identified in this paper to hopefully support this link.

4.4.5 How are these cytokines implicated in POEMS syndrome neuropathy?

Several cytokines in this pathway activate T and B cells, which in other causes of immune-neuropathy are thought to lead to infiltration, resulting in inflammation, demyelination and axonal pathology.[210,271] However, histopathological samples of nerve biopsies in POEMS syndrome seldom include immune cells, and in fact would point towards an alternative diagnosis if more than a few were seen.[17,18,84] Endothelial hyperplasia, blood nerve barrier leak, intravascular platelet adhesion and microthrombi, ischemia and oedema are more consistent findings of POEMS syndrome and appear to be related to the direct effects of circulating cytokines on the blood nerve barrier, with secondary pathogenic effects to the nerve resulting from an alteration in the endoneurial microenvironment. Of the cytokines studied, TNF-α is the only which has been clearly demonstrated to display pathogenic effects to nerve tissue, being toxic to myelin.[271] The effect of other circulating cytokines is unknown, however are thought to potentially have directly neurotoxic consequences as hypothesised above and illustrated in figure 4.5. Utilisation of PCR to detect RNA expression of cytokines in the peripheral nerves of POEMS syndrome cases may uncover specific immunological

changes at the level of the nerve which may enable a greater understanding of the pathological processes that occur, resulting in the histopathological findings which are already described. A disadvantage of this technique is that the presence of RNA does not always accurately reflect protein levels, and that it is difficult to identify the cellular sources of such cytokines unless specific cell types were first isolated. Since neither serum nor PCR cytokine analysis allows for identification of cytokine-producing cell types, immunohistochemistry can overcome this limitation. This can detect the structural localisation and types of cytokine producing cells. This may be of significant benefit when considering the role of cytokines in both disrupting the blood nerve barrier, and in neuronal pathology. Although histopathological specimens are useful to display the result of immune mediated damage to the blood nerve barrier and peripheral nerves, further work to study the impact of individual cytokines on such structures in 'real time' would be of significant utility. To explore this, I have formed a collaboration with Professor Alison Llyod's group, the MRC Laboratory for Molecular Cell Biology who are using a combination of animal studies with light and electron microscopy techniques to study the structure, function and regulation of the blood nerve barrier, and some of this work will be discussed in chapter 5.

Figure 4.5: Updated schema of POEMS syndrome pathogenesis



5. The inflammasome of POEMS syndrome

5.1 Introduction

Cytokines are fundamental to immune system signalling and activation and are probably key pathological drivers in POEMS syndrome. However non-cytokine protein markers may signify important pathways or bioproducts of the inflammatory cascade which are not immune mediated but provide important information about mechanisms of disease and underlying pathogenesis. Changes in these potential biomarkers can be ascertained by proteomic analysis. Proteomics involves the application of technologies for the global identification and quantification of proteins present in a cell, tissue, fluid or organism.[276] Proteomic analysis may therefore provide insight into other key biological processes involved in POEMS syndrome not previously discovered, that may have important contributions to the disease manifestation.

The following chapter introduces the fundamentals of proteomics and reviews the literature of proteomic studies in POEMS syndrome and related conditions. Experimental data is then presented studying the proteome of POEMS syndrome in disease, treatment and relapse. Conclusions are drawn from this data to provide further detail to underlying pathological processes occurring in POEMS syndrome and how they relate to clinical disease.

5.1.1 The study of proteomics using mass spectrometry

Proteomics-based technologies are used to detect diagnostic markers, understand pathological mechanisms and interpret functional protein pathways in disease. Proteomics can also assist in early diagnosis, prognosis, and monitoring disease. Characterisation of the proteome, that is the protein complement of the genome, includes the expression, structure, function, interactions and modifications of proteins at any developmental stage.

The first techniques which sought to purify and study proteins were chromatography based, such as ion exchange, size exclusion and affinity chromatography.[277] To analyse selected proteins, directed techniques such as ELISA and western blotting can be used, but limit analysis to few proteins. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis (2-DE) are techniques which separate complex protein mixtures, but rely on further probing to identify individual proteins.

Liquid chromatography coupled to mass spectrometry has become an indispensable technology in the identification of proteins in complex biological mixtures. Mass spectrometry is a high throughput technique which measures the mass-to-charge ratio (m/z) of gas phase ions.[278] Mass spectrometers consist of an ion source that converts analyte molecules into gas-phased ions, a mass analyser that separates ionized analytes based upon their m/z ratio, and a detector which then detects the number of ions at each m/z value to quantify.[279] The development of techniques which allow the

formation of molecular ions of intact biomolecules- electrospray ionisation (ESI) and matrix assisted laser desorption/ ionisation (MALDI) make polypeptides capable for mass spectrometric analysis.[280] In proteomic research, four types of mass analysers are commonly used; quadrupole (Q), ion trap, time of flight (TOF) and Fourier-transform ion cyclotron resonance (FRICR) mass analysers. Each analyser varies in physical principles and analytical performance and are discussed in detail elsewhere.[278]

Mass spectrometers can be used in several different ways to characterise the proteome. Two main analytical streams, the 'top down' and 'bottom-up' approaches are most commonly used.[281] Top down relies on the analysis of intact proteins by mass spectrometry, with characterisation through the fragmentation of proteins within the mass spectrometer followed by the measurement of the fragmented ions. This method enables the study of secondary and tertiary protein structures and protein complexes. In contrast, bottom-up analysis first digests proteins into smaller peptides, and the mass spectrometer is then used to identify the sequence of these peptides through fragmentation. The identified peptide sequences are reassigned to the proteins they originated from to characterise them. This allows for rapid, robust, high throughput qualitative and quantitative analyses of protein compositions. Bottom-up proteomic techniques were utilised for this project.

An overwhelming set of acquisition modes and data query strategies are available in bottom-up mass spectrometry. Two acquisition modes are used in this thesis and therefore discussed; data-dependent acquisition (termed shotgun or discovery proteomics) and targeted-data acquisition.[281]

5.1.1.1 Shotgun proteomics

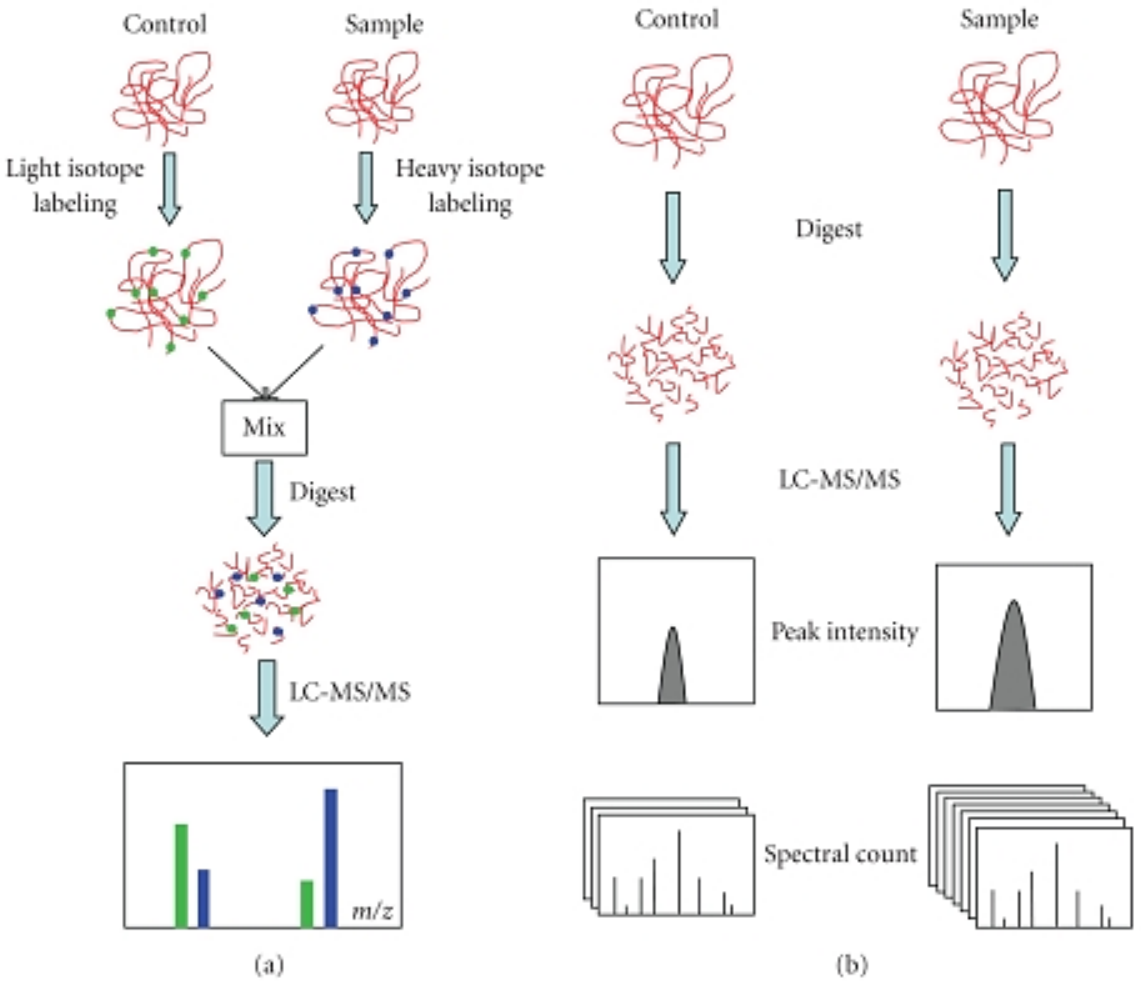
The development of "shotgun" proteomics refers to the use of bottom-up proteomic techniques in studying the entire protein expression of complex biological samples using a combination of liquid chromatography (LC) combined with mass spectrometry. This can be done using either 'labelled' or 'label-free' techniques. Labelled proteomic analysis relies on the fact that isotope labelled compounds are identical to their natural counterparts other than by mass, and therefore proteins can be labelled, isolated and measured.[282] Such techniques are very useful when comparing protein changes in complex samples, for example pre and post treatment. However, most labelling based approaches are time consuming and require complex sample preparation, and specific quantification software.[278] Also, most technologies only allow up to eight samples to be compared at the same time. For this reason, label-free quantitative proteomics have been developed to achieve faster, cleaner and simpler to quantify results.

Label-free proteomics involves sample preparation including protein extraction and digestion, followed by peptide separation. This can be done first at the protein level, using various fractionation processes such as electrophoresis, chromatography and

isoelectric focussing, all of which reduce sample complexity. This is followed by peptide separation by liquid chromatography (LC or LC/LC which involves two different separation mechanisms for better separation efficiency). LC separates peptides based on hydrophobicity using an organic solvent, such as acetonitrile. Another separation technique employed by the mass spectrometry equipment utilised in this study was ion mobility. This separates gas phase ions based upon their interaction with a collision gas within an electric field based on their respective size, shape and charge, providing better separation of samples compared to conventional methods. [283] This technique is also matrix and chromatography independent, ensuring reproducibility, unlike retention times, which can shift. Following peptide separation, analysis using mass spectrometric peptide identification and quantification is performed[283].

The quantification of proteins is performed using two techniques; measuring the ion signal intensity of peptide precursors associated with a given protein (peptide peak areas), and secondly counting the total number of fragmentation spectra that map to peptides of a given protein (called spectral counting).[284]

Figure 5.1: Isotope labelling versus label-free proteomics



(a) *Shotgun isotope labelling method: Labelling of proteins with light and heavy stable isotypes in the control versus sample, which are then combined and analysed by LC-MS. Quantification is calculated on the intensity ratio of the isotope labelled peptide pairs. (b) Label-free proteomics, in which control and disease samples are subject to LC-MS analysis, and quantification based on comparing the peak intensity or the spectral count of the same peptides.*

In mass spectrometry, an ion with a particular m/z is recorded to have a reproducible signal intensity (the peptide peak intensity) which correlates with concentration. Therefore, increased concentrations of injected peptides for electrospray ionisation correlate with increased peak areas. Once the peak areas of all identified peptides of a protein are combined, these will correlate linearly to the concentration of the input protein.

Spectral count, defined as the total number of fragmentation spectra identified for a protein, has gained acceptance as a practical, label free, semi quantitative measure of protein abundance in proteomic studies.[284] Relative protein quantification is achieved by comparing the MS spectra of trypsin digested peptides from the same protein across each of the LC-MS samples. An increase in protein results in an increase in the number of its proteolytic peptides. Large proteins contribute more peptide/ spectra than small proteins, and thus normalisation is required to standardise the effect of protein length on spectral count. This allows for a comparison of abundance of individual proteins in complex mixtures. [280]

A number of bioinformatic tools aid in automated label-free analysis for comparative liquid chromatography mass spec (LC-MS). Most packages follow the same process of data analysis with normalisation, time alignment, peak detection and matching, identification and statistical analysis.[282] The Waters(Milford, MA) mass spectrometry label free proteomics methodology employed in this study assign the fragment ion spectra to their associated precursor ion peaks using advanced software algorithms, so

that all the information necessary to identify each compound of interest is collated. Precursor and fragment spectra are then aligned according to retention times and linked together. Once this comprehensive dataset is generated it must be interrogated manually to determine the presence and concentrations of relevant molecules.

Shotgun proteomic strategies are efficient in sorting hundreds or even thousands of proteins involved in any given number of biological states, but often fall short of providing clinically relevant, practice-changing information if performed in isolation because of the complexity of information received. A major caveat of proteomics is that digested and fragmented peptides are represented relative to the abundance of each protein in the sample, causing the most abundant proteins to be identified more reliably while possibly less abundant but potentially relevant proteins can be overlooked. For this reason, a more targeted, hypothesis-driven approach which takes known biological data into account should be conducted alongside this broader way of studying the proteome. [285]

5.1.1.2 Targeted data acquisition

Targeted proteomics data acquisition has become the method of choice for the validation of protein candidates in independent, potentially large sample cohorts that were originally detected by high-throughput discovery type data dependent acquisition (shotgun) analyses. [286] The advent of targeted techniques mitigates the problem of recognition and quantification of low-abundance proteins inherent to shotgun

methodology, while allowing better quantitative representation of proteins of interest. Targeted assays can also add multiplexing capabilities that far exceed most immunological assays. This is often performed through a process of selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) using specific MS instruments like the triple quadrupole analyser.[285] The first quadrupole is pre-programmed to isolate peptide ions of a particular mass (the parent ion) to obtain a population comprising mostly the intended species. The second quadrupole acts as a collision chamber to fragment the selected peptides to yield product ('daughter') ions whose signal intensities are indicative of the abundance of peptide in the sample, before the third quadrupole filters further by m/z and analyses.[287] Only compounds which meet both these criteria i.e. specific parent and daughter ions corresponding to the mass of the molecule of interest are isolated and measured. By filtering all other ions that flow into the mass spectrometer, the experiment gains sensitivity whilst maintaining exquisite accuracy. Calibration curves can be created by spiking known amounts of purified peptides of the target protein into samples and comparing the precursor and fragment peaks.[288] This technique is best applied for consistently quantifying the presence of known peptides rather than discovering new ones. It is important to select peptides with amino acid sequences unique to the protein of interest (proteotypic peptides) to obtain an accurate representation of protein abundance. Since targeted proteomics is no more than a method for monitoring the precursor and fragment ions of previously selected peptides, the proteins under investigation must be well known beforehand with a good selection of proteolytic peptides to enable high sensitivity and specificity. Information used to select candidate peptides can be of various sources, making use of previous findings in the literature or from screening shotgun-based proteomic assays

Mass spectrometry often seeks to identify novel, reliable biomarkers to predict clinical outcome in disease in easily accessible patient biofluids such as plasma, serum, saliva, urine or faeces. [289] Serum and plasma are particularly attractive sources, however proteome characterisation of such heterogeneous, complex samples with a large dynamic range in protein concentrations provide a degree of limitation.[289] Targeted approaches are more suitable for this endeavour due to the ability of highly sensitive and specific quantification. Despite this, identification of novel biomarkers for clinical application remains a significant challenge. For example, among the FDA-approved mass spectrometry measured cancer biomarkers, nine exist for serum only, and six for either plasma or serum.[290]

5.1.2 Proteomics in POEMS syndrome, related plasma cell dyscrasias and neuropathies

Mass spectrometry has not been used before to study the proteome of POEMS syndrome. As is the case in most POEMS research, one can look to studies in multiple myeloma as a framework for study design and methodology, or to compare or identify differences in results. Zhang *et al* used liquid chromatography with mass spectrometry (LC/MS) to compare serum of myeloma patients to healthy controls and identified 22 proteins differentially expressed between the two groups including serum amyloid A, vitamin D binding protein isoform-1 precursor, plasma kallikrein and apolipoprotein A-1. [291] Apolipoprotein A-1 was lower in the sera of MM patients compared to healthy controls. This protein promotes efflux of cholesterol from cells and reduced expression is seen in other malignancies such as ovarian cancer.[292] Vitamin D binding protein was lower in MM patients and this protein is known to reversibly bind to and transport

vitamin D to target cells. Serum amyloid-A (SAA) was higher in MM patients, and has also been found to be higher in MM thalidomide non-responders. SAA is an acute phase protein that is associated with circulating high-density lipoproteins and modulates lipid trafficking and immune responses, and is the precursor protein in AA amyloidosis.[293] Plasma kallikrein was also elevated, and this participates in the surface-dependent activation of blood coagulation and inflammation.[291] The authors suggested that the biomarkers are mostly associated with the complement and coagulation cascade, lipid metabolism and extracellular matrix remodelling, all of which are known to be disrupted in myeloma pathogenesis. In many respects these data were superficial and require more in-depth analysis. Wang *et al* have used MALDI-TOF mass spectrometry of serum in myeloma patients compared to other plasma cell dyscrasias and healthy controls, and through the use of a computerised biomarker analysis decision tree, identified three biomarkers which correctly classified patients as myeloma to non-myeloma; unfortunately their decision tree was unable to satisfactorily differentiate myeloma from other plasma cell dyscrasias.[293] Although the authors did not attempt to classify the three m/z peaks identified in the study, they note that one of their markers (marker 2, 11.68kDa) appeared very similar to that of the previously identified serum amyloid A1 (which is 11.68kDa), and again highlight this as being a potentially relevant protein in myeloma diagnosis.

Stratifying particular disease features to ascertain biomarkers associated with severity or progression has been utilised as a methodology. Dowling *et* al studied serum from myeloma patients with and without bone lesions, and identified three potential protein biomarkers associated with bone disease, a feature of myeloma infiltration. [294] These

were complement C4-A and serum paraoxonase/arylesterase (PON1), both raised in bone disease, and apolipoprotein B-10 which was reduced in bone disease. C4 and PON1 were confirmed to be significantly associated with bone disease in a validation cohort. The authors discussed that complement may play a role in bone development and regeneration, which may explain why C4, a major protein of the classical cascade, may be significant. PON1 was suggested to provide a protective mechanism against oxidative stress on bone formation associated with osteoclastic resorption to try to balance bone remodelling.

Utilising therapies as disease activity 'knock downs' may also help identify pathogenic markers. For example, Hsieh *et al* adopted LC/MS to determine the plasma proteome of 10 myeloma patients pre, and 24 hours following, bortezomib therapy. [295] The plasma levels of two efficacy response markers (apolipoprotein C-I and its truncated isoform apolipoprotein C-I') were significantly more abundant in non-responsive patients compared to responders. Functionally, both markers are thought to be important regulators of lipid metabolism, however the link between lipid dysregulation and treatment resistance is not known.

Very few studies have investigated the proteome of inflammatory neuropathies. One study by Tumani *et al* in 2009 used two-dimensional gel electrophoresis of CSF from patients with CIDP (n=11) and controls (n=11) and analysed any protein spots which showed a significant difference using mass spectrometry.[296] Ten proteins were upregulated in CIDP (two transferrin isoforms, alpha-1 acid glycoprotein 1

precursor, apolipoprotein A IV, two haptoglobin isoforms, transthyretin (TTR), retinol binding protein and two isoforms of proapolipoprotein) and one protein was downregulated (integrin beta 8). A further validation experiment compared one candidate protein, TTR in patients with CIDP, healthy controls and 19 patients with GBS. Several of the protein markers were thought to not be disease specific to CIDP. For example, haptoglobin, transferrin and proapolipoprotein have been described in other studies investigating the CSF proteome in inflammatory and neurodegenerative diseases. [297–299] Haptoglobin is an acute phase protein with antioxidant properties, enhanced locally at sites of inflammation. Thus the authors postulate that either such proteins are common pathological substrates for a range of neurological disease processes, or otherwise happen to be comparatively easy to detect, while other disease specific proteins evade detection. TTR however has been identified in three independent studies analysing the CSF proteome in GBS.[300–302] Most studies observed TTR to be downregulated in GBS, while it was upregulated in CIDP in this study. The pathomechanism for this phenomenon in GBS and CIDP is unclear. TTR is a carrier protein for thyroxine and vitamin A, and is a negative acute phase protein. Mouse studies suggest TTR enhances nerve regeneration,[303] and thus was postulated to be associated with neuronal regeneration in the course of chronic inflammation of peripheral nerves.

A similar study in GBS was conducted by Jin et al, comparing CSF samples from GBS and healthy controls separated by 2D electrophoresis and analysed by mass spectrometry. [300] This study identified haptoglobin, apolipoprotein A-IV and PRO2044 (an unnamed protein) to be increased in GBS cases, and TTR, apolipoprotein E (ApoE) and fibrinogen

to be decreased. Haptoglobin and Apolipoprotein A-IV were considered to be related to the inflammatory processes involved, with recruitment of lymphocytes and monocytes. TTR, was reduced, again demonstrating its role as a negative acute phase protein. Another study by Yang *et al* identified cystatin C, transthyretin, apolipoprotein E and heat shock protein 70 to be reduced, with haptoglobin, α-1 antitrypsin, apolipoprotein A-IV and neurofilaments to be elevated, consistent with the study by Jin. The similarities in proteins expressed across studies provides a degree of validity to the results from these studies. However, once again a limitation of the findings may be that such proteins are high abundance non-specific markers of neuronal (or even non-neuronal) disease. Apo E appears to have a significant role to play in GBS, and is often abnormal in mass spectromic analysis. ApoE has multiple functions, studied in lipid metabolism, cerebrovascular disease and neurodegeneration. Non-lipid related properties of ApoE include suppression of T cell proliferation, regulation of macrophage function, facilitation of lipid antigen presentation by CD1 molecules to natural killer T cell and inhibition of blood derived inflammatory cells across the BNB. [304]

As discussed above, the proteome of POEMS syndrome is entirely unknown. Mass spectrometry is an appealing methodological technique compared to standard immunological assays due to the ability to study the entire proteome in an unrestricted manner without predefined targets. Although POEMS syndrome pathogenesis is thought to be broadly cytokine driven, this technique may uncover proteins involved in, or a result of, the pathological mechanisms of disease. Further multiplex assays can then also be performed to study a broad range of proteins at a high sensitivity and specificity. Knowledge of the functional properties of relevant identified proteins may also uncover

pathological processes inherent to the various organ systems involved, for example the discovery of neurofilament would be a marker of neuronal damage. This chapter presents data studying the proteome of POEMS syndrome, and compares that to related disease groups in order to uncover disease processes or biomarkers specific to POEMS syndrome.

5.1.3 Hypotheses

1. Proteins which are pathological markers of disease can be detected in POEMS syndrome and differ from that of related plasma cell dyscrasias, neuropathies and healthy controls.
2. The functions of such proteins indicate disease processes inherent to POEMS syndrome pathogenesis.
3. Levels of POEMS related proteins correlate with disease severity.

5.1.4 Aims

1. Perform unlabelled shotgun proteomics on serum of patients with POEMS syndrome, CIDP and healthy controls to identify potential POEMS related markers.
2. Run targeted mass spectrometry assay to identify potential inflammatory markers related to POEMS pathogenesis not previously identified through routine immunoassay.
3. Identify whether levels of POEMS-related protein markers indicate or correlate with markers of disease activity.

5.2 Methods

5.2.1 Study participants

Label free proteomic analysis was performed on five cases with pre-treated POEMS syndrome, five cases of CIDP and five healthy controls. The targeted analysis studied 45 matched pre and post treated POEMS cases, and 15 cases of matched relapse sera. 25 CIDP and 25 myeloma disease controls were selected, as were 25 healthy controls. The inclusion criteria for selection of POEMS cases are documented in section 3.5.2.1.

The five POEMS syndrome cases for label-free analysis were selected as 'classical' and severe presentations of POEMS with over 8 features at diagnosis, high VEGFs (>2000pg/ml) as an indication of active disease, and with samples collected within the previous 12 months. Cases of CIDP and multiple myeloma were selected using the inclusion criteria in section 4.2.1 and lack of prior treatment. Disease and healthy control samples were selected to be matched in age and sex distribution to the POEMS cases.

5.2.2 Sample collection

Blood was collected into serum tubes, centrifuged at room temperature at 3000 rpm for 5 minutes. Serum was separated and aliquoted immediately, then frozen at -80 ^{0}C until use.

5.2.3 Untargeted mass spectrometry

Serum samples were thawed and 10 ul sample was pipetted into a Top 12 abundant protein spin column resin slurry (ThermoFisher, MA, USA). The column was capped and inverted several times to suspend the resin in solution before being incubated with end-over-end mixing for 60 minutes at room temperature. The column was placed in a 2ml Eppendorf collection tube and centrifuged at 1000xg for 2 minutes. The 500ul filtrate then contains the serum sample with the top 12 most abundant proteins removed in 10mM PBS, 0.15M NaCl, 0.02% azide, pH 7.4 solution. Protein precipitation was performed by adding three volumes (1500ul) of ice cold acetone, followed by vortexing and freezing at minus 20 degrees for one hour. This sample was then centrifuged at 13000xg for 10 minutes to form of a pellet, and the supernatant was removed and discarded. The pellet was washed with 500ul of ice-cold acetone and recentrifuged, with the supernatant again being discarded. The pellet was then reconstituted in 100ul of water, vortexed to dissolve and frozen on dry ice before being freeze-dried overnight.

To each dried protein sample, 20ul of digest buffer (6M urea, 2M thioreua, 2% ASB14, 100mM Tris-HCL pH 7.8) was added and left to shake for one hour. Next, 1.5ul of dithioerythriol (DTE) (30 mg/ml) was added to break disulphide bonds, and then shaken at room temperature for one hour. Subsequently 3.0ul of iodoacetamide (IAA) was added and shaken, protected from light, for 45 minutes to carboamidomethylate all cystine residues. To this solution, 155ul of dd H_20, and 20ul of 0.1ug/ul trypsin gold (Promega, WI, USA) were added followed by incubation overnight at 37 ^oC to digest e the proteins.

Peptides then underwent fractionation. Samples were diluted with 200ul 0.2% ammonia to adjust to a final concentration of 0.1%. Solid phase extraction cartridges (ISOLUTE C18, 1ml) were equilibrated with 2x700ul 50% acetonitrile (ACN), 0.1% ammonia and then 2x700ul H_20, 0.1% ammonia. Peptide samples were loaded and allowed to drip by gravity. The cartridges were then washed with 500ul of 0.1% ammonia before the peptides were eluted in fractions with 250ul of solution containing increasing ACN percentage, at 3%, 6%, 9%, 12%, 15%, 18%, 21%, 24%, 30% and 50% containing 0.1% ammonia twice, collecting samples in the same plastic tube each time. Solvents were evaporated and the dried peptides stored at -20 °C until use.

Peptides were analysed in fractions using a SYNAPT G2-Si (Waters, Manchester) Mass Spectrometer coupled to a nanoACQUITY UPLC (Waters, Manchester) as previously described.[305] Raw data was processed using Progenesis Qi software (Nonlinear dynamics, UK). Proteins were identified against a downloaded UniProt human reference proteome. Data was searched with fixed modifications of carboamidomethylation of cysteines, dynamic modifications of deamidation of asparagine/glutamine and oxidation of methionine, one missed cleavage sites, and a false discovery rate set at 1%. Only protein identifications with >95% confidence and more than one peptide were used to determine significance in protein expression between groups.

5.2.4 Multiplexed targeted mass spectrometry

Serum samples were thawed and 30ul aspirated for analysis. 10ul of 30ug/ml yeast enolase protein (ENO1- Sigma) was added to each serum sample to act as an internal standard accounting for variation in sample preparation. Sera were precipitated with 80ul of 3M ammonium sulphate to allow for the detection of medium and low abundance proteins, then incubated at room temperature for 10 minutes prior to centrifugation at 1690 x g for 10 minutes to separate a supernatant from the protein pellet. The protein pellet was washed with 150ul 1.8M ammonium sulphate.

Proteins were solubilised using 40ul digest buffer containing 6M urea, 2M thiourea, 2% ASB-14 and 100mM Tris pH 7.8 and shaken until the protein pellet was dissolved. To reduce disulphide bonds, 180ug of dithioerythriol was added and shaken for 60minutes, and 432ug iodoacetic acid added to prevent disulphide bonds reforming, with a further 30 minutes of shaking. 330ul MilliQ water was added to dilute the concentration of urea, and 2ug trypsin (Promega) was added to digest the proteins into tryptic peptides. Samples were then incubated at 37 °C in a water bath for at least 12 hours.

In order to remove salts, a solid phase extraction (SPE) was performed on a 100 mg Bond Elut C18 96 well plate (Agilent, Santa Clara, CA, United States). Prior to SPE clean up, the samples were adjusted to a concentration of 0.1% trifluoroacetic acid (TFA). The SPE beds were washed with 1 mL 60% acetonitrile (ACN), 0.1% TFA before equilibration by two 1 mL aliquots of 0.1% TFA. The samples were loaded onto the beds and salts were washed away by the addition of 1 mL 0.1% TFA before elution of the

desalinated samples by two 250 µL aliquots of 60% ACN, 0.1% TFA. Solvents were evaporated using a SpeedVac (Eppendorf).

Before mass spectrometric analysis, the samples were reconstituted in 50 µL 3% ACN, 0.1% TFA. Utilising a Waters Acquity Ultra Performance Liquid Chromatography system (Waters), 4 µL of sample was injected onto a Waters 50 mm CORTECS® UPLC® C18 1.6 µm column fitted with a 5 mm VanGuard column of the same chemistry, operating at 45 °C, for chromatographic separation. The mobile phase consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in ACN, pumped at a flow rate of 0.6 mL min-1. The starting conditions of 3% B were kept static for 0.1 minutes, before initialising the linear gradient utilised to elute and separate the peptides over 7.7 minutes to 40% B. B was thereafter linearly increased to 80% over 0.2 minutes and held for 1 minute to wash the column before returning to the initial conditions followed by equilibration for 1 minute prior to the subsequent injection. The LC system was coupled to a Waters Xevo-TQ-S triple quadrupole mass spectrometer for multiple reaction monitoring (MRM) detection in positive electrospray ionisation mode. The capillary voltage was set to 2.8 kV, the source temperature to 150 °C, the desolvation temperature to 600 °C, the cone gas and desolvation gas flows to 150 and 1000 L hour-1 respectively. The collision gas consisted of nitrogen and was set to 0.15 mL min-1. The nebuliser operated at 7 bar. The cone energy was set to 35 V and the collision energies varied depending on the optimal settings for each peptide. Three injections were performed for each sample.

After acquisition, the data were imported to Skyline open-source software (https://skyline.ms/project/home/software/Skyline/begin.view). The quantifier and qualifier ions were inspected and compared to a pooled sample spiked with increasing

amounts of synthetic peptides to ensure that the correct peaks were identified and integrated. The relationships between quantifier and qualifier ions were evaluated for consistency across the samples and the most abundant peptide ions were chosen for quantitation. The analyte quantifier areas were normalised to spiked internal standard to render ratios. The relative response of each analyte to the response of the internal standard provided a normalised ratio of peptide to spiked standard for each sample as a relative comparator.

5.2.5 Statistical and bioinformatic analysis

Exported data were analysed using Excel and Graphpad Prism, with results displayed using Graphpad Prism. Analysis of the affected pathways was conducted with Ingenuity Pathway Analysis IPA, Qiagen). STRING (Search Tool for the Retrieval of Interacting Genes) database was used to determine known and predicted protein interactions. Targeted peptide data from each condition were compared against healthy control data using Mann Whitney U. Pearson and Spearman correlations were used to determine relationships between biomarkers (Pearson) or risk scores (Spearman). Peptides from matched pre and post-treatment POEMS samples were compared using Wilcoxon matched pairs analysis.

5.3 Results

5.3.1 Label free mass spectrometry analysis

5.3.1.1 Study participants

The median age of the pre-treated POEMS cohort was 60 years (range 46-76). Of the CIDP cohort, all were new diagnoses and treatment naïve, with a median age of 53 (range 38-76). Healthy control median age was 58 (range 44-81). Each group comprised of three males and two females.

5.3.1.2 POEMS serum proteome compared to CIDP and healthy controls

In total, over 250 proteins were detected in 10ul of depleted serum by label free quantitative proteomic analysis. Analysis of the POEMS syndrome proteome revealed at least 1.5-fold or more increased expression of 23 proteins and reduced expression of 14 when compared to age and sex-matched healthy controls. Table 5.1 lists the proteins involved, including the confidence score (a measure of confidence of accurate protein identification). CIDP samples displayed increased expression of 17 proteins, and decreased expression in two compared to healthy controls.

The volcano plot in figure 5.2 provides a visual summary of the degree to which these proteins are altered firstly in POEMS syndrome compared to healthy controls, and secondly in CIDP compared to healthy controls as a disease control. These diagrams plot both fold change and statistical significance, and thus the upper, outermost peptides are of most interest. Firstly, more proteins significantly differed from healthy controls in

POEMS syndrome than they did in CIDP (37 versus 21), and with greater log fold differences overall (see table 5.1). The two proteins upregulated and downregulated with the highest significance values were labelled on the volcano plots (see figure 5.2). For POEMS syndrome, Serum amyloid-A4 and Haemoglobin subunit beta were most upregulated, and Plasminogen-like protein A and Immunoglobulin heavy constant γ2 most downregulated. For CIDP, Serum amyloid-A4 and Apolipoprotein E were upregulated, and Galectin-3-binding protein and Plasminogen-like protein A downregulated.

Table 5.1: POEMS syndrome proteomics serum profiling

POEMS Upregulated						
Gene	Name	Peptide count	Unique peptides	Confidence score	ANOVA (p)	Fold change
SAA1	Serum amyloid A-1	8	7	42.0	0.036	35.03
IGLL1	Immunoglobulin lambda-like polypeptide 1	3	2	8.5	0.048	18.30
CFL1	Cofilin-1	2	2	10.8	0.018	13.86
EIF3G	Eukaryotic translation initiation factor 3 subunit G	3	2	5.8	0.004	9.12
IGHV5	Immunoglobulin heavy variable 5-51	2	2	13.5	0.028	4.39
LUM	Lumican	11	9	67.5	0.019	4.24
APOE	Apolipoprotein E	38	36	107.4	0.003	3.62
HBB	Hemoglobin subunit beta	9	6	47.6	0.003	3.48
PZP	Pregnancy zone protein	17	10	151.1	0.005	3.40
C5	Complement C5	121	111	672.2	0.024	3.38
ACTB	Actin_ cytoplasmic 1	4	3	29.1	0.048	3.32
SAA4	Serum amyloid A-4 protein	6	6	30.4	0.002	3.25
SERPINA10	Protein Z-dependent protease inhibitor	4	4	25.4	0.040	3.18
FCN3	Ficolin-3	10	10	64.3	0.046	3.03
MRTFA	Myocardin-related transcription factor A 1	3	3	8.0	0.028	2.99
IGKV3	Immunoglobulin kappa variable 3-20	2	2	15.9	0.020	2.72
FBLN1	Fibulin-1	2	2	23.5	0.019	2.54
C1S	Complement C1s subcomponent	22	19	151.2	0.006	2.47
C8A	Complement component C8 alpha chain	14	14	132.8	0.007	2.35
SERPINA1	Alpha-1-antitrypsin	230	225	562.3	0.014	2.16
SERPINC1	Antithrombin-III	88	84	366.4	0.016	1.74
SERPINF2	Alpha-2-antiplasmin	49	46	235.7	0.040	1.70
C1QB	Complement C1q subcomponent subunit B	13	13	76.7	0.016	1.66
PON1	Serum paraoxonase/arylesterase 1	21	19	153.5	0.046	1.57

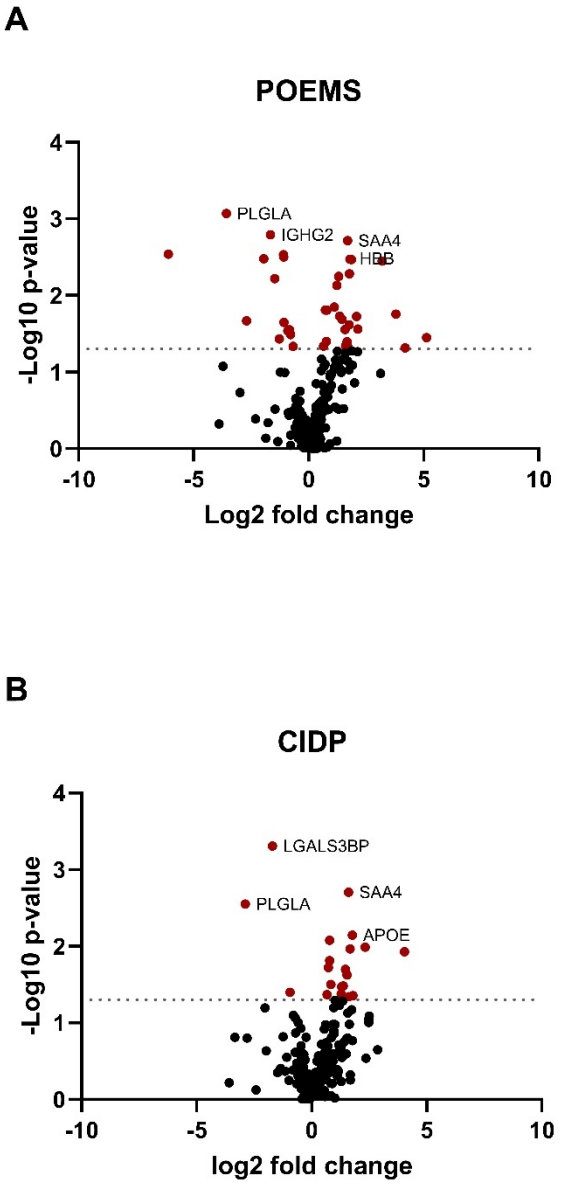
POEMS Downregulated						
Gene	Name	Peptide count	Unique peptides	Confidence score	ANOVA (p)	Fold change
KRT87P	Putative keratin-87 protein	3	3	5.7	0.003	-68.31
PLGLA	Plasminogen-like protein A	7	4	30.0	0.001	-11.85
LCP1	Plastin-2	3	3	16.6	0.021	-6.46
LGALS3BP	Galectin-3-binding protein	22	22	134.3	0.003	-3.86
IGHG2	Immunoglobulin heavy constant gamma 2	64	43	349.9	0.002	-3.15
ATRN	Attractin	14	13	86.6	0.006	-2.78
IGHV3	Immunoglobulin heavy variable 3-23	3	2	23.6	0.037	-2.42
CPN1	Carboxypeptidase N catalytic chain	5	3	29.4	0.003	-2.13
APOA2	Apolipoprotein A-II	65	64	158.2	0.003	-2.12
F10	Coagulation factor X	6	6	38.9	0.022	-2.09
PROS1	Vitamin K-dependent protein S	20	18	119.3	0.029	-1.88
AGT	Angiotensinogen	58	56	190.2	0.028	-1.77
CFP	Properdin	7	5	36.6	0.032	-1.72
IGFALS	Insulin-like growth factor-binding protein complex acid labile subunit	12	10	78.3	0.046	-1.59

CIDP Upregulated						
Gene	Name	Peptide count	Unique peptides	Confidence score	ANOVA (p)	Fold change
CFL1	Cofilin-1	2	2	10.843	0.012	16.453
EIF3G	Eukaryotic translation initiation factor 3 subunit G	3	2	5.752	0.010	5.017
CRTAC1	Cartilage acidic protein 1	2	2	9.088	0.044	3.477
APOE	Apolipoprotein E	38	36	107.435	0.007	3.428
PZP	Pregnancy zone protein	17	10	151.143	0.011	3.192
SERPINA10	Protein Z-dependent protease inhibitor	4	4	25.422	0.046	3.066
SAA4	Serum amyloid A-4 protein	6	6	30.384	0.002	3.040
THBS1	Thrombospondin-1	33	31	175.838	0.024	2.902
MRTFA	Myocardin-related transcription factor A	3	3	8.008	0.020	2.785
PPBP	Platelet basic protein	11	11	20.966	0.033	2.596
C8A	Complement component C8 alpha chain	14	14	132.845	0.034	2.473
APCS	Serum amyloid P-component	35	33	118.416	0.042	2.439
GPLD1	Phosphatidylinositol-glycan-specific phospholipase D	5	5	22.694	0.105	1.895
KNG1	Kininogen-1	75	68	305.803	0.031	1.792
HRG	Histidine-rich glycoprotein	54	50	153.589	0.015	1.724
PON1	Serum paraoxonase/arylesterase 1	21	19	153.519	0.008	1.717
AFM	Afamin	80	74	252.735	0.019	1.652
GSN	Gelsolin	55	55	348.534	0.043	1.590

CIDP Downregulated						
Gene	Name	Peptide count	Unique peptides	Confidence score	ANOVA (p)	Fold change
PLGLA	Plasminogen-like protein A	7	4	30.044	0.003	-7.365
LGALS3BP	Galectin-3-binding protein	22	22	134.259	0.000	-3.252
GP5	Platelet glycoprotein V	2	2	11.593	0.040	-1.921

Differential expressed serum proteins pertaining to five POEMS syndrome cases to 5 healthy controls (top two tables) and five CIDP cases to 5 healthy controls (bottom two), quantified by label-free mass spectrometry. Confidence score, significance and fold change presented.

Figure 5.2: Volcano plot of the proteome in POEMS syndrome and CIDP



Volcano plot depicting log2 fold change of peptides against -Log1 p-values for POEMS syndrome vs healthy controls (A) and CIDP vs healthy controls (B). Outermost peptides with largest log2 fold change, uppermost most significant.

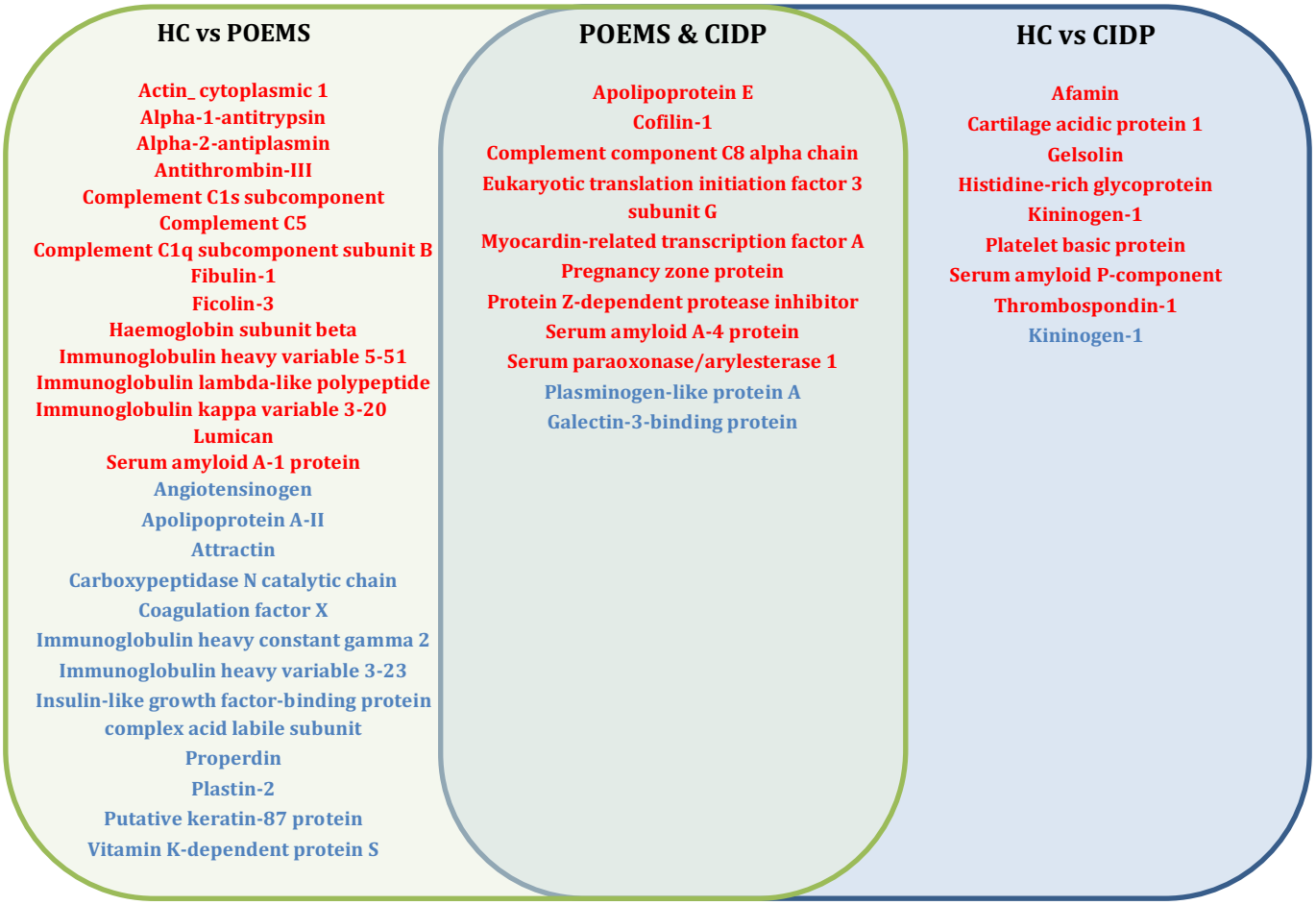
When comparing peptides between POEMS syndrome and CIDP sera, there were only six proteins which differed significantly between the two conditions (with a p-value <0.05 and a normalised fold change of >1.5), with an increase in only two proteins in POEMS syndrome; Immunoglobulin heavy constant alpha 1, which was also increased 18.3-fold compared to healthy control samples, and Metalloproteinase inhibitor 1 which was not significantly different when compared to healthy control samples. Four were reduced compared to CIDP; Putatative keratin-87 protein, Apolipoprotein A-II, Phosphatidylinositol-glycan-specific-phospholipase D, and Plasminogen-like protein A.

The Venn diagram in figure 5.3 demonstrates the proteins that were altered in both POEMS syndrome and CIDP compared to healthy controls (HC), and which proteins differed in one or other disease compared to HC. This demonstrates there were more differentially expressed proteins specific to POEMS syndrome compared to healthy controls (n=28) than there were for CIDP (n=9), and a significant proportion of proteins with similar increases or decreases compared to healthy controls in POEMS and CIDP (n=11), which could explain the lack of markers which differentiated between the two conditions.

Studying the proteins that were equally activated or deactivated in both POEMS syndrome and CIDP (which may be specific to neuronal damage), the only one of potential neurological relevance is Cofilin-1. This protein has been demonstrated previously to be in part responsible for regenerative axonal growth in damaged peripheral nerves, and therefore may be released in peripheral nerve damage to

encourage neuronal recovery. [306] Other peptides such as Serum amyloid A-4, Apolipoprotein E and complement signify similar immune mediated pathologies, the relevance of which will be discussed in detail below.

Figure 5.3: Venn diagram of unlabelled proteomic analysis of POEMS, CIDP and healthy control samples.



Red and blue coloured metabolites indicate significant elevation or reduction compared to healthy controls ($p < 0.05$). Peptides arranged alphabetically.

In order to gain insights into the biochemical pathways in POEMS syndrome that arise from the differentially expressed proteins detected in the serum, an Ingenuity Pathway Analysis (IPA, QIAGEN Inc) was conducted.[307] The results of this analysis are shown in

figure 5.4. This displays the most significant pathways involved in POEMS syndrome. The significance threshold was set to $p<0.05$, expressed as a -log(p-value) of 1.3 (using Fisher's Exact test). The significance indicates the probability of association of molecules from the dataset with the canonical pathway by random chance alone. The colour coding of bars is based on the pathway's z-scores, which is a statistical measure the programme uses to make predictions of activation or inhibition. It does this by comparing the expected state of a gene or protein (being up or downregulated) predicted to occur if the pathway were activated, comparing this to the actual fold change. White bars represent pathways in which the evidence for activation and inhibition are equal. Gray bars represent pathways in which the activity pattern could not be calculated due to lack of sufficient evidence in the programme's database.

The most significantly activated pathway is the Liver X Receptors and Retinoid X Receptors (LXR/RXR) pathway due to the differential expression of angiotensinogen, apolipoprotein A2 and E, paraoxonase 1, serum amyloid A4, serpin family A member 1 and serpin family F member 2. This pathway is involved in the regulation of lipid metabolism and in inflammation. Interestingly, the coagulation system was involved, with coagulation factor X and protein S reduced, and serpin family A member 1, serpin family member C 1 and serpin family F member 2 increased. Due to the increased expression of the serpins, the coagulation pathway is thought to exhibit decreased activity, which would be suggestive of an anti-coagulant effect.

The program can additionally identify diseases and functions expected to increase or decrease given the differentially expressed proteins in the dataset. This provides a -log p-value, representing the measure of the likelihood that the association between a set of molecules in the dataset and a related disease is due to random association. The higher the value, the more likely the association is thought to be significant. Figure 5.5 lists these functions against the -log p-value, with only functions in which the -log p-value was over 5 (to highlight key functions). The most significant functions were that of humoral response, inflammation, organismal injury (which involves blood clots, thrombi, myocardial infarction, vaso-occlusion and stroke), and haematological disease (many of which were related to coagulation). Neurological disease was also highlighted, with a number of CNS conditions such as stroke and Alzheimer's disease, but also with more relevant conditions such as Amyotrophic Lateral Sclerosis and progressive motor neuropathy.

Figure 5.4: Ingenuity Pathway Analysis of biochemical pathways involved in POEMS syndrome

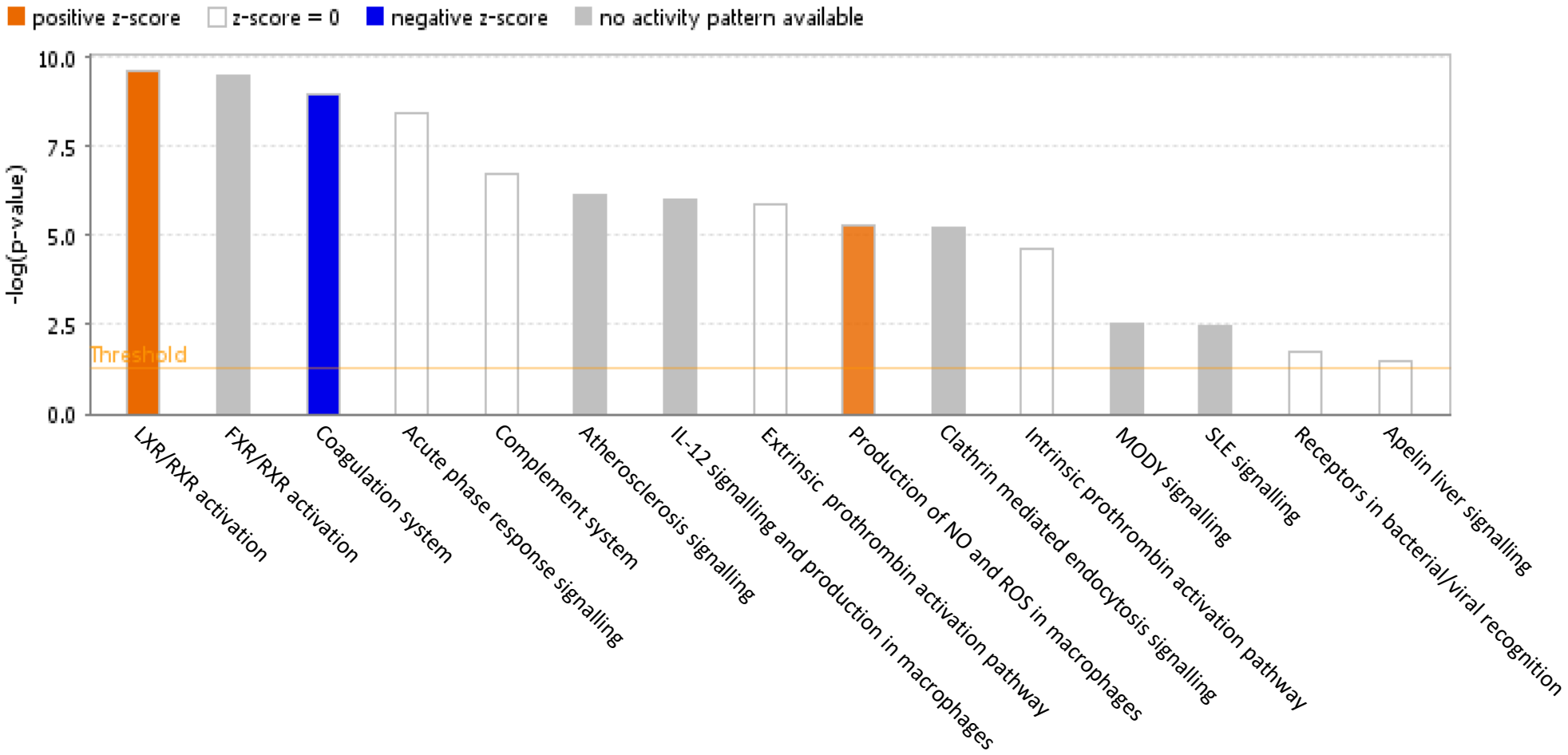
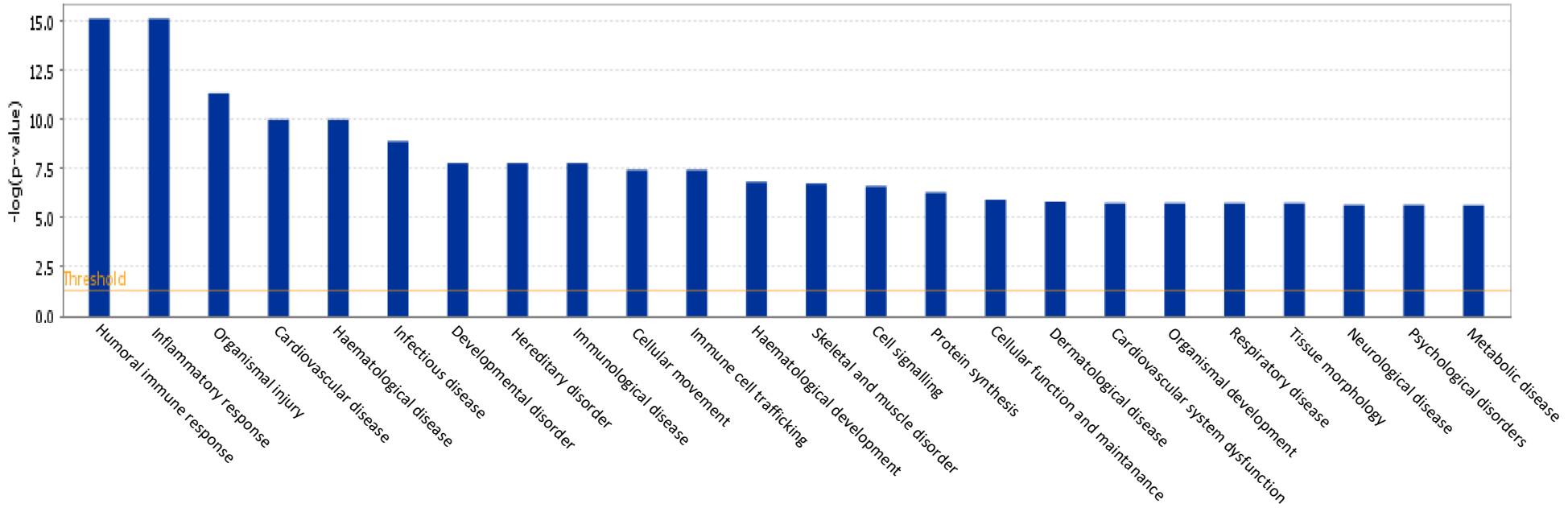
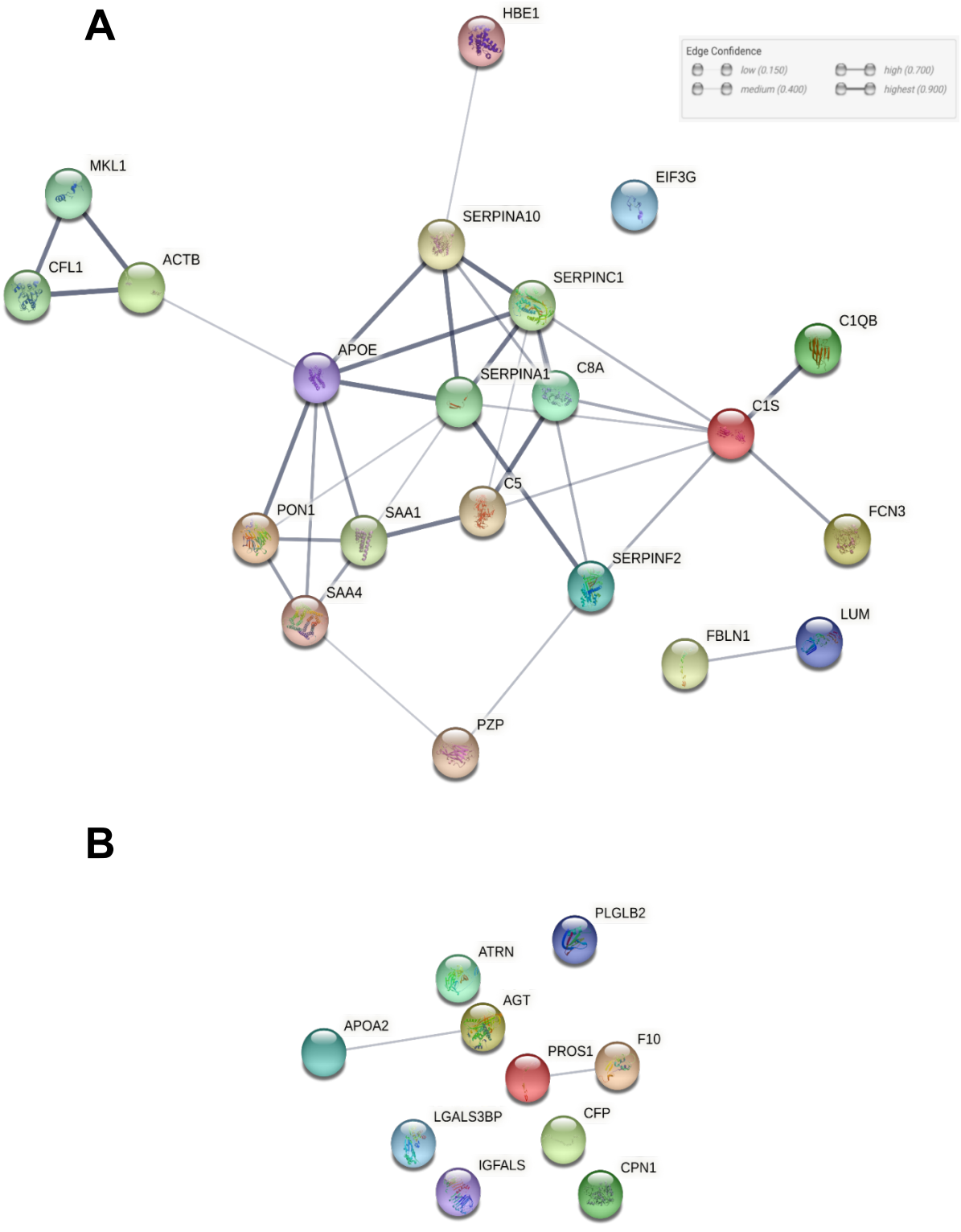


Figure 5.5: Ingenuity Pathway Analysis of disease pathways involved in POEMS syndrome



Protein associations and networks from the list of proteins with significantly changed abundances between POEMS and healthy controls were analysed using STRING (Search Tool for the Retrieval of Interacting Genes), a database of known and predicted protein interactions.[308] Networks of proteins are created through linking proteins to a variety of sources including interaction databases, text mining, experimental data, and genetic interactions. Two analyses are displayed, figure 5.5a demonstrates functional associations between the upregulated proteins in POEMS syndrome compared to healthy controls, and 5.5b displays downregulated proteins. The legend displays the strength of association between proteins.

Figure 5.6: STRING diagram of unlabelled proteomic analysis of POEMS syndrome peptides both upregulated (A) and downregulated (B)



Gene and peptide names detailed in table 5.1. A= upregulated proteins, B = downregulated. Note SERPINA1= α-1-antitrypsin, SERPINF2= α-2-antitrypsin, SERPINC1= antithrombin III, SERPINA10= protein-z-dependent protease inhibitor

Of the upregulated proteins, there were several connected pathways, resulting in an enrichment p-value of <1.0e-16, signifying that the proteins have more interactions among themselves than that expected for a random selection of proteins of similar size drawn from the genome. STRING analysis of the downregulated proteins demonstrate far fewer links, with an enrichment p-value of 0.07, indicating proteins are more randomly collected, and thus the cluster of downregulated proteins in POEMS syndrome compared to healthy control are potentially less relevant than those upregulated. Strengths of enrichment effects are also included in this analysis, that is the ratio between the number of proteins in the network annotated with a term compared to the expected number to be annotated with the same term in a random network of the same size. Biological processes with high strength scores are documented below (note genes and names documented in table 5.1):

- Complement activation (C5, C8A, C1S, C1QB, FCN3)
- Inflammatory response (SAA1, SAA4, SERPINA1, SERPINF2, SERPINC1, C5)
- Blood coagulation (HBB, SERPINC1, SERPINA10, SERPINA1, SAA1, FBLN1)

When studying the network analysis of downregulated proteins in POEMS syndrome, fewer pathways were involved, however those that were also appeared to be of relevance and consistent with the findings above:

- Regulation of complement activation (PROS1, CFP, CPN1)
- Regulation of the inflammatory response (AGT, PROS1, CFP, CPN1)
- Formation of fibrin clot (PLGLB2, PROS1, F10)

5.3.2 Multiplex targeted spectrometry analysis

A predefined multiplex mass spectromic assay that was curated by the UCL translational mass spectrometry laboratory was used in this study which measured 80 peptides known to be involved in immune mediated inflammation, the presumed mechanism of disease in POEMS syndrome. Following peak picking and selecting appropriate standards, 20 peptides were identified as bad hits and removed, with 60 peptides providing appropriate data for analysis. Table 5.2 summarises all the proteins found to be significantly altered (>2-fold) in the disease groups compared to controls in this study. The largest range of peptides differed in the POEMS syndrome compared to healthy controls than in the CIDP or multiple myeloma group. Peptides altered in each group are discussed below.

5.3.2.1 POEMS syndrome specific peptides

The MRM-based mass spectral analysis demonstrated that 21 biomarkers were significantly altered (15 elevated, 6 suppressed) in the pre-treated POEMS serum compared to controls. Biomarkers with the largest increase were intracellular adhesion molecule 1 (ICAM1), CD5 antigen-like protein (CD5L), amyloid-beta A4 protein (APP) and C1- inhibitor; 9.5, 7.8, 7.1 and 4.7- fold higher in the POEMS group respectively compared to the healthy controls. Six biomarkers were suppressed in POEMS syndrome compared to controls, the most significant being neuropilin 1, pyruvate kinase, basic fibroblast grown factor 2 and immunoglobulin heavy constant epsilon, approximately -8.5, -4.3, -4.1 and -4.0 fold reduced compared to controls.

There were 12 proteins which were specifically altered in POEMS syndrome serum compared to healthy controls (with >2-fold difference and $p<0.05$), in which levels between disease groups (CIDP and myeloma) and healthy controls did not differ. These peptides are highlighted in table 5.2. Elevated proteins were C1-inhibitor, heat shock protein 7c, cyclin-dependent-kinase-like 5, cytochrome c, Collagen Type VI Alpha 3 Chain, Complement C3 and Remodelling and Spacing Factor. The potential functional properties of such proteins will be discussed in detail. Figure 5.7 demonstrates the relative proportions of such biomarkers in POEMS syndrome compared to that of disease matched cases and healthy controls.

None of the identified peptides correlated with ONLS level, indices of the haematological monoclonal plasma cell burden pre-treatment serum VEGF levels or with the calculated prognostic risk score detailed in chapter 2.3.6.

5.3.2.2 POEMS syndrome and disease match related peptides

Intracellular adhesion molecule-1, Neurofilament medium, Tubulin alpha-4A chain and CD5 antigen-like peptide were significantly raised above controls in both POEMS syndrome and in CIDP, but not in myeloma which may reflect neural tissue damage. [309] ICAM-1 has been demonstrated to be involved not only in inflammation, but also in cell recruitment during Wallerian degeneration following peripheral nerve injury, leading to macrophage recruitment and supporting neurite outgrowth. [309] Multiple myeloma shared similar peptides to POEMS syndrome, with 3 peptides equally up- or downregulated compared to healthy controls. PLD3 (Phospholipase D Family Member

3) is upregulated significantly in POEMS syndrome and downregulated in myeloma compared to controls, which may be considered a useful biomarker to distinguish POEMS cases from myeloma. Peptides which were unique to myeloma were Fibrinogen alpha chain and Extracellular superoxide dismutase which would distinguish myeloma from both POEMS and CIDP. Figure 5.8 demonstrates peptides shared between POEMS and CIDP, and POEMS and myeloma.

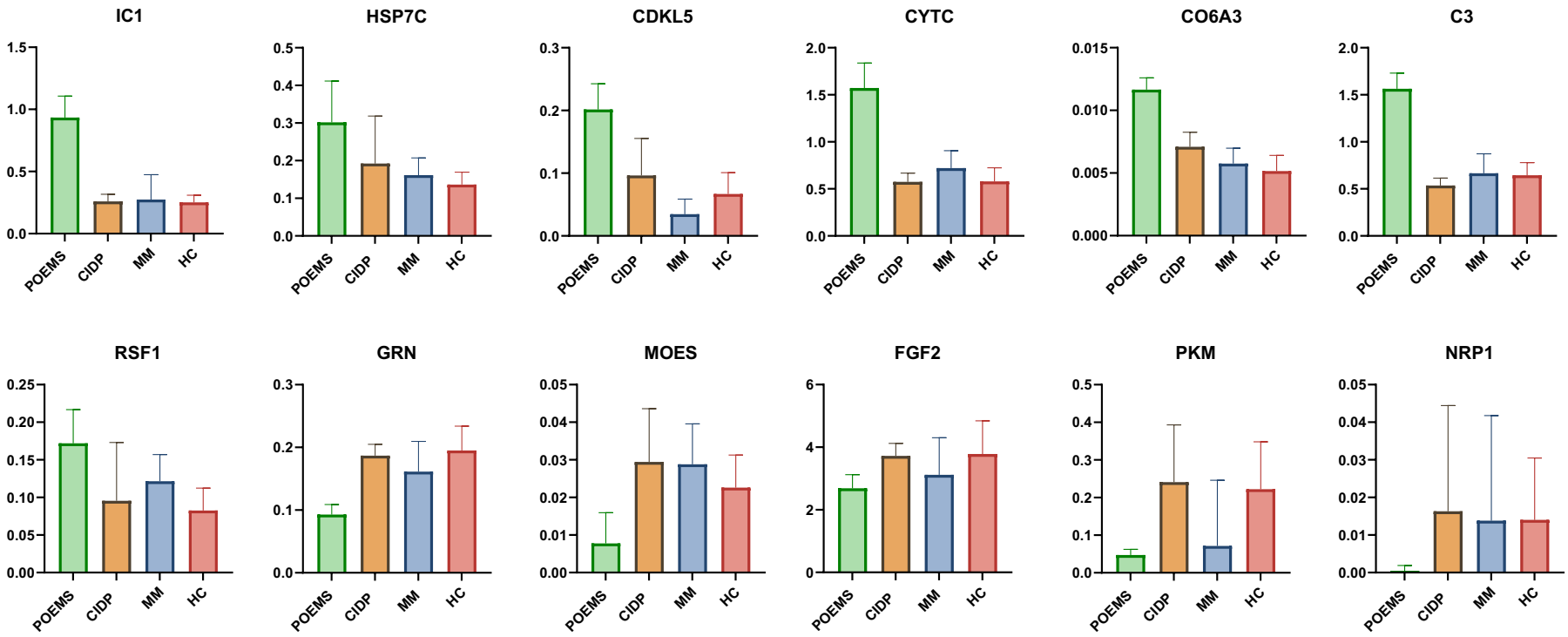
Table 5.2: Targeted proteomic mass spectrometry protein markers in POEMS syndrome, CIDP and Multiple Myeloma

POEMS syndrome		CIDP		Multiple myeloma	
Gene name	Fold change (p-value)	Gene name	Fold change (p-value)	Gene name	Fold change (p-value)
ICAM1	9.5 (0.02)	**ICAM1**	10.1 (0.0001)	**APP**	3.6 (0.02)
CD5L	7.8 (<0.0001)	**NEFM**	6.3 (0.001)	**FGA***	1.9 (0.015)
APP	7.1 (<0.0001)	**TUBA4A**	5.1 (<0.02)	**PLD3**	-2.6 (0.0009)
IC1*	4.7 (<0.0001)	**CD5L**	2.8 (<0.0001)	**SOD3***	-2.9 (<0.0001)
HSP7C*	3.8 (<0.0001)			**HS90B**	-3.2 (<0.0001)
NEFM	3.5 (0.0002)			**IGHE**	-6.2 (0.02)
CDKL5*	2.9 (<0.0001)				
PLD3	2.6 (<0.0001)				
CYTC*	2.6 (<0.0001)				
COL6A3*	2.4 (<0.0001)				
C3*	2.4 (<0.0001)				
TUBA4A	2.1 (<0.0001)				
RSF1*	2.0 (<0.0001)				
GRN*	2.0 (<0.0001)				
MOES*	-2.4 (0.001)				
HS90B	-2.6 (<0.0001)				
IGHE	-4.0 (0.006)				
FGF2*	-4.1 (0.0002)				
PKM*	-4.3 (<0.0001)				
NRP1*	-8.5 (<0.0001)				

* denotes a protein which is differentially expressed in that condition alone

ICAM1: Intercellular Adhesion Molecule 1, CD5L: CD5 Antigen-Like, APP: Amyloid Beta Precursor Protein, IC1: C1 inhibitor, HSP7C: heat shock protein 7c, CDKL5: cyclin-dependent-kinase-like 5, PLD3: Phospholipase D Family Member 3, CYTC: cytochrome c, CO6A3: Collagen Type VI Alpha 3 Chain, C3: Complement C3, IGHA2: Immunoglobulin Heavy Constant Alpha 2 , TUBA4A: Tubulin Alpha 4a, RSF1: Remodelling And Spacing Factor , GRN: Granulin precursor, MOES: Membrane-Organizing Extension Spike Protein, HS90B: heat shock protein 90b, IGHE: Immunoglobulin Heavy Constant Epsilon, FGF2: Fibroblast Growth Factor 2, PKM: pyruvate kinase, NRP1: Neuropilin 1, SOD3: Superoxide Dismutase 3, NEFM: Neurofilament Medium.

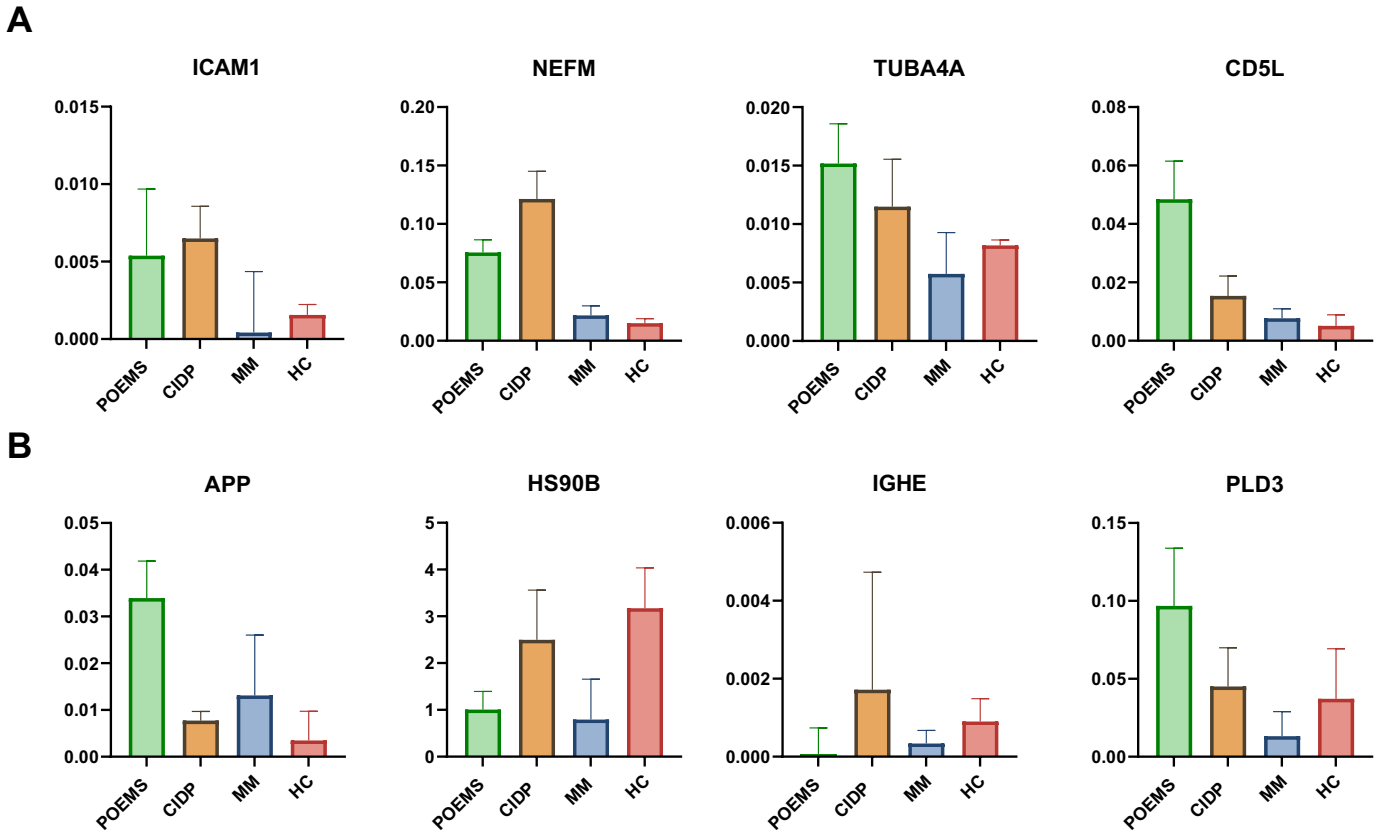
Figure 5.7: POEMS specific protein markers



IC1: C1 inhibitor, HSP7C: heat shock protein 7c, CDKL5: cyclin-dependent-kinase-like 5,CYTC: cytochrome c, CO6A3: Collagen Type VI Alpha 3 Chain, C3: Complement C3, RSF1: Remodelling And Spacing Factor , GRN: Granulin precursor, MOES: Membrane-Organizing Extension Spike Protein, FGF2: Fibroblast Growth Factor 2, PKM: pyruvate kinase, NRP1: Neuropilin 1

Graphs depicting individual peptide (with peptide code centred above the graph) compared as a ratio of abundance in each condition (POEMS syndrome, CIDP, MM- multiple myeloma, and HC- healthy control) against an internal standard control, and thus data is comparative across groups. All peptides in this figure were significantly altered (>2-fold difference, p<0.05) in POEMS syndrome cases compared to healthy control (HC) samples, with no significant difference between disease matched cases (CIDP and MM) compared to controls.

Figure 5.8: Common markers between POEMS syndrome and disease controls



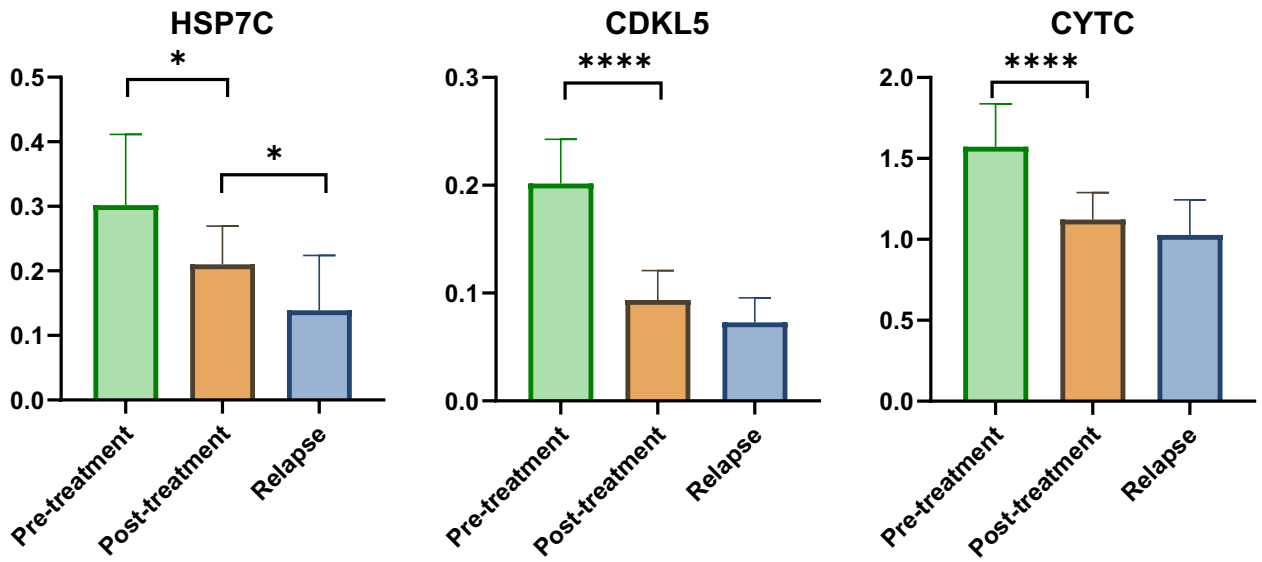
ICAM1: Intercellular Adhesion Molecule 1, NEFM: Neurofilament Medium, TUBA4A: Tubulin Alpha 4a, CD5L; CD5 Antigen-Like peptide, APP: Amyloid Beta Precursor Protein, HS90B: heat shock protein 90b, IGHE: Immunoglobulin Heavy Constant Epsilon, PLD3: Phospholipase D Family Member 3.

*A= Each marker is significantly altered (>2-fold difference, p<0.05) in POEMS syndrome **and** CIDP compared to other groups. B= Each marker is significantly altered (>2-fold difference, p<0.05) in POEMS syndrome **and** myeloma compared to other groups, other than PLD3 in which levels are significantly upregulated in POEMS and downregulated in myeloma. Note Y axis represents ratio of biomarker to internal standard control and thus data is comparative across groups.*

5.3.2.3 POEMS markers in response to treatment and relapse

Peptide markers in response to therapy and relapse are displayed in figure 5.9. Of the 12 peptides found to be specifically up- or downregulated in POEMS syndrome compared to healthy controls, three were subsequently significantly downregulated following treatment ($p<0.005$); these were heat shock protein 7c ($p=0.02$), cyclin-dependent-kinase-like 5 ($p<0.0001$) and cytochrome c ($p<0.0001$). Peptide levels were also compared from post-treatment samples and those obtained at relapse, where the only significant alteration was in heat shock protein 7c ($p=0.02$). However, the level of this peptide reduced further compared to the post-treatment sample rather than increasing again as might be expected.

Figure 5.9: Peptides significantly altered in POEMS syndrome at pre-treatment compared to post treatment and relapse



HSP7C: heat shock protein 7c, CDKL5: cyclin-dependent-kinase-like 5, CYTC: cytochrome c

Peptides in POEMS syndrome in which levels significantly reduced following treatment, and corresponding changes upon relapse. Note Y axis represents ratio of biomarker to internal standard control and thus data is comparative across groups.

5.4 Discussion

This chapter uncovers a number of previously unknown proteins associated with POEMS syndrome, providing some further insights regarding the underling pathogenesis.

Untargeted mass spectrometry enables an unbiased exploration of proteins altered in disease states, which may reveal important and potentially unrecognised biological pathways which are disrupted or activated. Targeted multiplex panels allow the determination of a large number of complex peptides within a sample in relatively short time at high sensitivity. Each methodology however has limitations which will be discussed in further detail at the end of this chapter. Utilisation of this technique was thought to complement that of the previous scientific methodologies utilised in this thesis, providing a broader investigation into disease biomarkers than cytokines alone. Proteomic studies were also hypothesised to be potentially of more use in identifying specific damage products rather than underlying disease drivers (such as cytokines) which may serve as biomarkers of disease for prognostication, such as markers of neuronal, renal, liver, or cardiac disease.

The design of mass spectromic studies are fundamental to the research question or hypothesis posed. For example, comparing the proteomic profile of the condition of interest against well characterised disease matched and healthy controls should firstly portray the distinct proteomic fingerprint of the condition, whilst also identifying important similarities and differences which may illustrate the role such proteins have

in specific aspects of disease. As seen in the introduction, comparing proteomic profiles of the same disease but studying different phenotypes may be another technique to identify key proteins or physiological pathways activated or inhibited specific to that particular disease process. Lastly, comparing proteomics of active disease cases versus quiescent/ post-treatment cases and relapse may also signify markers of disease or suppression.

Analysing the 'big data' mass spectrometry creates, and interpreting that in light of the clinical information is fundamental to translating such scientific discoveries into clinically useful findings. Although a multitude of bioinformatic methods exist, the mechanistic understanding of how such proteomic alterations disrupt biological processes leading to disease often requires not only complex computational methods, but also in-depth human analysis. The discussion below attempts to interpret the proteomic data whilst taking into account our current understanding of POEMS syndrome biology to identify potential markers of disease activity, propose fundamental disease pathways, and highlight areas of further exploration.

5.4.1 Untargeted mass spectrometry

Firstly, comparing the proteins identified through mass spectromic analysis in POEMS syndrome compared to healthy controls will be discussed, to interpret how such proteins may cause disease, before comparing against other disease matched conditions. The untargeted mass spectrometric panel in this study identified over 250 proteins from 10ul depleted serum, of which 38 were significantly altered (>1.5 fold and to $p<0.05$) compared to the serum of healthy controls.

Serum amyloid-A1(SAA1) had the greatest fold change and Serum amyloid A-4 (SAA4) protein the greatest statistical significance when compared to healthy controls. The serum amyloid A (SAA) family are typical factors of inflammation. The initiation of the acute phase response mechanism against infectious and inflammatory insults relies upon endogenous cytokines, broadly interleukins, TNF-α and interferon, produced by macrophages and leukocytes. These cytokines induce acute phase proteins, the most abundant of which is SAA from the liver. [310] The complete biological functions of SAA remain incompletely understood, but they are thought to be diverse. SAA has been demonstrated to have chemoattractant properties, stimulate further immune cell secretion of inflammatory cytokines and possess pro-angiogenic properties. [310,311] SAA has also been implicated in chronic inflammatory diseases including multiple sclerosis, neuromyelitis optica, rheumatoid arthritis, type II diabetes, atherosclerosis and malignancies, however whether SAA is causative or a consequence of these diseases is unknown. [312–314] It is likely serum amyloid A1 and A4 are produced in POEMS syndrome as a result of prolonged and excessive immune cytokine mediated inflammation. Persistently high serum SAA is known to promote AA amyloidosis, in which AA protein derived from SAA folds into extremely hydrophobic beta-sheets which aggregate to oligomers, forming insoluble proteolysis resistant fibrils in multiple organs. [315] Secondary AA amyloidosis is not reported other than in a single POEMS case in the literature and is not a finding we have experienced in our POEMS clinic practice; so does not appear to be a common finding, [316] but as it may be a very rare feature of a very rare disease, the actual incidence may be comparable to that of AA amyloid in commoner conditions. Possibly of greater relevance, SAA1 and SAA4 have been demonstrated to

correlate with makers of blood hypercoagulability and the incidence of venous thromboembolism. [317,318] Venous and arterial thromboembolism were recorded as frequent complications of POEMS syndrome, as presented in section 2.3.2.13 and published in more detail elsewhere. [94] Levels of SAA were not higher in patients who had recorded venous and arterial thromboembolism, although serum sampling was not performed at the time of thrombosis which would be more appropriate to study this potential association, in particular studying the biomarkers longitudinally in cases whom develop thrombosis in order to determine if levels correlate leading up to the event.

In the blood, the vast majority (>95%) of liver derived SAA is bound to high-density lipoprotein (HDL), and inhibits the action of HDL. Since HDL mediates cholesterol transport, excess SAA may result in excess cholesterol in peripheral tissues. [319] Apolipoprotein E (ApoE) was also significantly upregulated in POEMS syndrome compared to controls, at 3.6 fold. In the circulation, apoE is part of several classes of lipoprotein including chylomicrons, very low density lipoproteins (VLDL) and HDL. Similar to SAA, apolipoprotein E is involved in the metabolism of triglyceride rich lipoproteins. Abnormalities in lipid metabolism have not been studied in this thesis as lipid disturbances have not been described until recently and were not thought to be intrinsic to POEMS pathogenesis. However, interesting new data has recently emerged demonstrating a characteristic lipid profile of POEMS syndrome compared to healthy controls from a lipidomic study of 24 POEMS syndrome patients in China. [320] The authors identified lipidomic markers correlating with VEGF and clinical risk scores, but

made no attempts to formulate a hypothesis regarding the role of lipids in POEMS pathogenesis.

Apolipoprotein E also has potential non-lipid related properties including suppression of T cell proliferation, regulation of macrophage function and modulation of inflammation and oxidation. [320–324] ApoE has been demonstrated to result in an imbalance of Th1/Th2 cell populations, an imbalance that may result in autoimmune disorders. This has been demonstrated in particular using EAN mice, whereby Th1 cytokines, such as IL-1β, IL-6, IL-12, IL-17, IL-23, TNF-α and INF-γ, predominate. [325] These cytokines recruit effector cells to the PNS leading to damage. ApoE knock-out mice display a combination of blood nerve barrier disruption and significant upregulation of the Th1 cytokines at both the mRNA and protein levels, suggesting ApoE functions to suppress immune activity and related damage.[326]

Observing the volcano plot in figure 5.2, Haemoglobin subunit beta (HBB) was also identified as being both substantially increased and differed significantly to that of healthy controls. HBB is the globin protein which makes up haemoglobin A (HbA) and does not appear to be immediately of relevance in POEMS syndrome. The protein is released from the bone marrow and thus may be a consequence of disorganised haematopoiesis, or may be produced in response to VEGF and hypoxic states, since it's functional role is to increase oxygen transport to tissues. Additionally, HBB is cleaved into spinorphin; spinorphin is an antagonist of the P2RX3 receptor which is involved in pain signalling, and thus is a regulator of pain and inflammation. [327] Whether this

process occurs in POEMS is unknown, and notably spinorphin wasn't an identified peptide in this study, but may have been at undetectable levels. Further study using mass spectrometry to measure levels of HBB and spinorphin could be conducted in patients with painful peripheral neuropathy in POEMS syndrome versus those that do not, and to those of CIDP (which is again often painless) to determine whether levels correlate with the presence and extent of pain.

There was also upregulation of the following proteins implicated in immunoglobulin production;

Upregulated

- Immunoglobulin lambda -like polypeptide 1 (18 fold)
- Immunoglobulin (Ig) heavy variable 5-51 (4.3-fold)
- Ig kappa variable 3-20 (2.9-fold)

Downregulated

- Ig heavy constant γ2 (-3.1-fold)
- Ig heavy variable 3-23 (-2.4-fold)

Such findings were specific to POEMS syndrome compared to healthy controls, and not seen in CIDP. Due to methodological limitations in terms of sample sizes for the non-targeted proteomic study, myeloma samples were not tested, and thus important comparisons not made.

The most consistent previously reported finding in POEMS syndrome is the expression of light chains being derived from the IGLV1 subfamily, specifically the IGLV 1-40 or the IGLV 1-44 germline sequences, thought to be secondary to antigen-driven selection. [328,329] However this has not been a consistent finding, with the dominant germline detected in only 8/24 cases in one study. [329] The mass spec data in this study revealed increased expression in an IGK (Ig kappa) variable domain, which is atypical for POEMS which is almost always lambda light chain restricted. Further analysis of this cohort of five cases reveals three demonstrated IgG lambda and two IgA lambda paraproteins. Although serum free light chain ratios were normal throughout, all cases had abnormally raised kappa light chains, with an average of 49mg/L (upper limit of normal is 19.3mg/L), including raised lambda chains also. Although monoclonal plasma cells typify POEMS diagnosis, histopathological data demonstrates a polyclonal response occurs, which may account for such findings in the mass spectrometry data, and the kappa light chain abnormalities seen in the blood. The Ig heavy variable 5-51 that was detected may be a product of restricted heavy chain variable usage in POEMS syndrome, although such findings have not been previously described in the literature. Studies have demonstrated IGHV2 and IGHV3 are over-represented in multiple myeloma which may differentiate it from POEMS syndrome. [330] This would require further mass spectrometry study, comparing POEMS and myeloma serum, possibly using larger numbers to identity similarities and differences in immunoglobulin peptides identified.

Immunoglobulin lambda-like polypeptide-1 (IGLL1) was 8.5-fold elevated in POEMS compared to healthy controls. IGLL1 does not appear related to immunoglobulin production per se, but is a preB cell receptor (also termed CD179b) found on the surface

of proB and preB cells, and is involved in transduction of signals for B cell proliferation and differentiation, allelic exclusion at the Ig heavy chain gene locus and promotion of Ig light chain gene rearrangements. Although the functional relevance in this protein is not known in POEMS syndrome, the above would suggest the peptide promotes and/ or is a marker of developing B cells, clonal or otherwise. Again, whether this B cell receptor drives aberrant lambda light chain production is entirely unknown. Further immunohistochemistry or flow cytology could be conducted to determine whether this receptor is present on the surface of B or plasma cells in the bone marrow of patients with POEMS syndrome.

Proteomic analysis of serum immunoglobulins by mass spectrometry has several challenges, which may substantially affect the validity of the findings described above. Firstly, to enrich low abundant proteins, the 'top ten' removal kit is used to remove the top ten abundant proteins, which make up 98% of the serum's overall protein composition. Immunoglobulins are one of these top ten proteins. This means that the immunoglobulins which have then been subsequently identified may purely be enriched immunoglobulins which have not been effectively removed in the sample preparation, or incorrectly identified proteins by mass spectrometry analysis. Secondly, the production of antibodies is complex. Immunoglobulins comprise of two identical heavy chains and two light chains. Each chain has a variable domain at the N-terminal end and a constant at its C-terminal. Genes that encode the Ig reside at three loci in the genome, IGH (heavy), IGK (kappa) and IGL (Lambda). [331] Each locus consists of variable (V), diversity (D - but only for IGH), joining (J) and constant (C) genes. Variable domain production occurs when one V, one D (IGH) and one J gene are somatically rearranged,

followed by the transcription of pre-messenger RNA containing the rearranged V-D-J and one IGHC (for heavy chains) or the rearranged V-J and IGKC or IGLC, with subsequent translation to create the heavy or light chain respectively. [332] The combination of such diverse chains provides impressive variability in the host antibody repertoire poised to recognise a suite of pathogen associated antigens. The presence of highly similar immunoglobulin proteins with interspersed segments of variable and conserved amino acid sequences generate recurring patterns in peptide mass spectra of V gene peptides, complicating the assignment of correct sequences to mass spectral data. Complete antibody sequences are not encoded in the germline, but assembled by DNA recombination, diversified within individual B cells. The typical strategy of constructing a reference database from the genome sequence is not useful for interpreting antibody-derived mass spectra. [333] More complicated methodologies have been constructed to overcome this limitation by high-throughput sequencing of immunoglobulin variable domains from an individual's B cell population to construct a sample-specific antibody sequence databases. [334] It is therefore likely that, although immunoglobulin heavy and light peptides have been identified in this study, they may be inaccurate or incorrect based on the methodological limitations described.

5.4.1.1 Pathway analysis of the POEMS proteome

5.4.1.1.1 The LXR/RXR pathway

Pathway analyses of mass spectromic data allows more complex bioinformatic calculation, to extract meaning from protein abundance data. The most significantly activated pathway was that of the Liver X Receptors and Retinoid X Receptors (LXR/RXR) pathway. Liver X receptors (LXRs) form functional heterodimers with

retinoid X receptors (RXRs) and regulate cholesterol, lipid and glucose metabolism. LXR/RXR activity is reported to have both anti-inflammatory and anti-angiogenic effects. [335] Studies have demonstrated expression and activation of LXRs in CD-4 lymphocytes, which reduces the secretion of Th-1 proinflammatory cytokines, including interferon γ, TNF-α and IL-2. [336] LXRs are also negative regulators of macrophage inflammatory gene expression, inhibiting expression of mediators such as IL-1β, MCP-1, MCP-9, cyclooxygenase-2 and IL-6.[335] It would therefore appear that high circulating levels of VEGF, IL-6 and other pro-inflammatory cytokines in POEMS syndrome may result in secondary activation of the LXR/RXR activation in an attempt to dampen the immune response and secondary tissue damage. LXRs also increase cholesterol transport and attenuate inflammation in macrophages, preventing the development of atherosclerosis within the blood vessel wall. Although numbers are small, 12% of our POEMS cohort experienced myocardial infarction, and 9% stroke (table 2.4). VEGF, chronic immune activation and inflammation, vessel wall atherosclerosis and hypercoagulability appear to contribute, and thus compensatory anti-inflammatory signalling through the LXR/RXR pathway may represent a protective mechanism.

5.4.1.1.2 Coagulation

The coagulation system was also significantly activated in POEMS syndrome compared to controls, however surprisingly most proteins identified were in-fact anti-coagulant in function. This was most likely due to the presence of the serpins as detailed in the model illustrated in figure 5.6, with SERPINA1 (α-1-antitrypsin), SERPINF2 (α-2-antiplasmin), SERPINC1 (Antithrombin-III) and SERPINA10 (protein Z-dependent

protease inhibitor, with downregulation of coagulation factor X. Plasminogen like protein A, a prothrombotic protein was downregulated 12-fold in POEMS syndrome compared to controls. The result of this pattern of coagulation factors on blood coagulability is unknown. It may be that regardless of the number of anti-coagulant proteins, the overall effect of significantly downregulating plasminogen like protein is a pro-coagulant effect, and the reason patients with POEMS syndrome develop clots. A significant limitation to the clotting data is the fact that all five serum samples for the untargeted mass spectromic panel were taken from patients on treatment with low molecular weight heparin. The five cases selected for this study were patients with known pre-treated POEMS syndrome, admitted to the ward for confirmation of disease and to be started on therapy. For this reason, blood samples could be collected and immediately taken to the laboratory for processing and storage without delay which was considered an advantage for mass spectromic and cytokine study. However, an unforeseen limitation is that all patients had already received treatment with low molecular weight heparin as part of their inpatient management. This can clearly have significant effects on clotting indices through activation of antithrombin which accelerates inhibition of factor X. This is likely to be why 'extrinsic prothrombin activation pathway' was another functional pathway discovered on bioinformatics analysis. CIDP samples were collected from patients attending daycare to start IVIG therapy and were therefore not on anticoagulation. For this reason, coagulation data may not truly reflect the pattern seen in POEMS syndrome, requires interpretation with caution and would require repeating in untreated patients. This could either be achieved by ensuring blood is taken from hospitalised inpatients prior to starting heparin therapy, or by studying blood profiles of patients collected via the outpatient clinic. Another limitation is the use of serum rather than sodium citrate anticoagulated

plasma. The development of a clot will have occurred in the bottle of serum samples, which will in itself can exhaust clotting factors, and thus provide inaccurate results when then tested on mass spectrometry. Serum was determined to be the most appropriate sample type in this study, however further mass spectrometry analysis should be conducted using anticoagulated plasma when studying the clotting aspects of POEMS syndrome which may provide more valid results.

5.4.1.1.3 Immune response and complement activation

The acute phase response and complement system were identified as being significantly altered in POEMS syndrome compared to healthy controls, however bioinformatic analysis was unable to determine whether such pathways would be predicted to be activated or inactivated. Prior knowledge of POEMS pathology would theorise the pathway was activated in disease. This provides further evidence to the underlying immune mediated pathogenesis of POEMS syndrome described in chapter 4. Complement C1 and C1q subcomponent, and C5 were upregulated. The complement system can be activated by antigen-antibody complexes as part of the classical pathway, or by complement C3 hydrolysis via the alternative pathway, or finally, antigens without the presence of antibodies via the mannose-binding lectin pathway. All pathways result in the activation of C3 which leads to production of the membrane attack complex, which creates a pore in cell membranes resulting in cell death. [337] Not only that, significant immune activation and modulation occurs from complement cleavage products alone. For example, complement C5 displays powerful biological properties in the recruitment of inflammatory cells such as neutrophils, eosinophils, monocytes and T lymphocytes, activation of phagocytes and release of granule based enzymes which all

contribute to innate immune function and tissue damage. [338] There are no data reported previously on the association of complement and POEMS syndrome. The mass spectromic data from this study suggest that both humoral and cellular immunity may be activated in POEMS syndrome, however the role of complement itself is less well defined. This may be relevant when modelling disease in POEMS syndrome (see chapter 6), where damage might only occur in the presence of complement, or possibly particular complement cleavage products.

Studying the IPA biochemical (figure 5.5) and STRING network analyses, humoral response, inflammation, haematological disease, cardiovascular disease and organismal injury (which involves blood clots, thrombi, vaso-occlusion and stroke in its list) were pathways most likely to be activated by the proteins which were discovered by mass spectrometry. Although these data are from a small cohort of patients (N=5), such disease features are recognised features of POEMS syndrome which provides some internal validity to the results, but also provides further detail into the key components that contribute to such manifestations. Further direct exploration should be performed through producing a POEMS specific targeted biomarker panel, amassing the proteins identified through this shotgun proteomic study and those identified in the literature to study such networks in more depth, quantitatively, and with larger patient numbers. Not only that, as mentioned before, different disease phenotypes can be included (i.e. those with different monoclonal dyscrasias, those with focal vs generalised disease, those with thrombosis vs those without, different neuropathy severities etc), or those pre and post treatment to explore how the proteomic profiles differ between groups.

5.4.1.2 Comparing POEMS syndrome proteome to CIDP

The aim of comparing the range of POEMS syndrome proteins to that of CIDP was to potentially identify similarities between the two which may indicate peripheral nerve specific markers. For example, if peptides related to neurofilament or peripherin were detected, it would be likely that such components were related to neuronal pathology.

Firstly, the overall number of proteins identified in POEMS syndrome (N=38) were greater when compared against CIDP (N=21) which is likely a reflection of the multi-system disease in POEMS syndrome, compared to what is considered a more focussed immunological myelin attack in CIDP. SAA4 and apoE were similarly significantly upregulated in both CIDP and POEMS syndrome. As mentioned previously, these proteins may be in response to systemic immune activation, however this is potentially less likely to be the case in CIDP which is not characterised by systemic hyperinflammation. ApoE has also been demonstrated to be synthesised by Schwann cells following nerve injury, with marked accumulation in the distal nerve stump hypothesised to facilitate cholesterol transfer in remyelination, promoting neuronal repair and regrowth.[339] It may therefore be that ApoE is a lipoprotein released in response to chronic inflammation, modulating the immune system and promoting neuronal repair. As such, increases of both peptides may reflect a combination of systemic hyperinflammation in POEMS syndrome or be more specific to the neuropathy itself.

Cofilin-1 was upregulated by 16-fold in CIDP and similarly by 13-fold compared to healthy controls in POEMS syndrome. This protein has been demonstrated previously to be in part responsible for regenerative axonal growth in damaged peripheral nerves, and therefore may be released in peripheral nerve damage to encourage neuronal recovery. [306] Within hours of sciatic nerve injury, levels of cofilin dramatically increase at the lesion site, and separately, elevating cofilin activity results in faster nerve growth and recovery. [306]

Disappointingly, there were less neuronal proteins identified through mass spectromic analysis than was hoped for. Notably, no peripheral nerve neurofilaments or demyelination markers were detected from the unlabelled mass spectrometry. This may be a result of testing blood serum, which includes the entire systemic circulation of proteins. Neuronal specific proteins, even if released in abundance from peripheral nerve damage, are unlikely to reach concentrations similar to the other proteins detected in this study. This illustrates the requirement of protein seperation prior to mass spectrometry, creating targeted platforms or novel highly sensitive immunoassays to study (such as that performed in chapter 3). This was evidenced by levels of neurofilament-medium being detected at higher levels in POEMS and CIDP serum compared to that of myeloma and healthy controls in the targeted mass spectromic study, which is discussed in more detail below. This demonstrates that neurofilament medium is clearly involved in neuronal pathogenesis of POEMS syndrome, however levels are far too dilute to be identified through an untargeted study.

Although several more proteins implicated in coagulation were identified in POEMS syndrome sera, Plasminogen-like protein A (PLGLA), the inactive precursor of plasmin was significantly downregulated compared to controls in CIDP (-7 fold) as it was in POEMS syndrome (-11 fold). PLGLA is a potent serine protease involved in the dissolution of blood clots. [340] Plasminogen deficiencies are typically associated with thrombotic conditions. Thrombosis is seen in CIDP, particularly in the context of IVIG therapy, [104] and therefore PLGLA may contribute to this process.

Although a number of other proteins were upregulated at an abundance in which they were detected upon untargeted mass spectromic analysis (such as Pregnancy Zone Protein or Eukaryotic Translation Initiation Factor 3 Subunit G (see factor 5.3)), such proteins do not appear to relate to the pathogenesis of POEMS syndrome or that of CIDP.

5.4.2 MRM-based Mass Spectral Analysis of the Inflammasome

5.4.2.1 Proteins significantly altered in POEMS syndrome compared to healthy controls

The MRM-based mass spectral analysis tested a total of 80 peptides which were thought to be implicated in immune mediated inflammation. This panel was used to identify the presence of specific inflammatory proteins in a large number of samples at high sensitivity. A major limitation of this experimental methodology was that the panel was predefined and created as a multiplex to explore biomarkers of COVID-19 disease mechanisms, details of which have been published elsewhere. [341] The panel focussed

broadly on immunity and inflammation, and was therefore considered to be a potentially useful multiplex panel in the study of POEMS pathogenesis. However, ideally a targeted biomarker panel would be created for proteins previously identified or implicated in the literature, and from developing the preliminary data gathered from an initial untargeted study. This however was not possible to achieve in this study. This meant that several proteins initially identified on the untargeted shotgun proteomic study were not included, and therefore the panel itself may not accurately capture POEMS pathogenesis. Regardless of this, significantly altered proteins detected through the targeted study are potentially important, and possibly novel, and may provide further insights to POEMS syndrome pathogenesis. Proteins with the highest fold changes in POEMS syndrome compared to that of healthy controls are detailed below (summarised in table 5.2), and their potential role in disease is discussed.

The intracellular adhesion molecule 1 (ICAM1) is a transmembrane glycoprotein belonging to the immunoglobulin superfamily and is expressed by several cell types, including leucocytes, fibroblasts and endothelial cells. Proinflammatory cytokines can markedly increase ICAM1 surface expression resulting in extensive cytoskeletal remodelling events which alter endothelial cell contractility and function resulting in transendothelial leukocyte migration to sites of inflammation.[337,342] Elevated levels have also been found in a number of malignancies including leukaemia, hepatocellular carcinoma, melanoma and small cell lung cancer,[343] and CNS disorders such as multiple sclerosis, experimental allergic encephalomyelitis and Alzheimer's disease.[344] In the CNS, ICAM1 is thought to disrupt the BBB, leading to increased permeability. Although not previously reported, it is therefore conceivable ICAM has a role in the blood nerve

barrier function in POEMS. Interestingly, ICAM1 was significantly upregulated in both POEMS syndrome (9.5 fold) and CIDP (10.1 fold), but not in myeloma. Blood nerve barrier breakdown is a key pathological process in immune mediated neuropathies, [90] which from the data in this study may be mediated by ICAM1. Myeloma does not directly cause neuropathy; neuropathy largely occurs as a secondary effect of drugs, nutrition, compression or amyloid accumulation. It is therefore possible that despite cytokines and other neurotoxic substances that exist in the serum of myeloma patients, such compounds cannot access the endoneurium, due the lack of ICAM1 upregulation in myeloma and maintained integrity of the blood nerve barrier, preventing the development of neuropathy. To study this further, immunohistochemistry of neuronal tissue samples should be performed staining for ICAM1. This data would also suggest that therapeutic target blockade of ICAM1 might provide neuroprotection in immune-mediated neuropathies.

CD5 antigen-like protein (CD5L) is a soluble protein expressed mostly by macrophages during inflammation. Expression is transcriptionally controlled by LXRs, and thus CD5L plays important diverse roles at the intersection between lipid homeostasis and the immune response, involved in processes such as infection, atherosclerosis and cancer. [345] This is achieved through prevention of leukocyte apoptosis, B cell proliferation and lipid metabolism. [346] The role CD5L has in POEMS syndrome is unclear.

Generation of the neurotoxic Aβ peptide from sequential amyloid precursor protein (APP) is the crucial step in development of Alzheimer's disease.[347] However, why APP was increased in both POEMS (7.1 fold) and myeloma (3.6 fold) is not understood. In addition, APP has not been identified in mass spectromic analysis of myeloma serum in previous studies in the literature and therefore may be incorrectly identified.

Twelve peptides were specifically up or down regulated in POEMS syndrome compared to healthy controls, that is, they were specifically altered compared to controls, whilst levels between disease groups and controls did not differ (see table 5.2 proteins with asterixis). On analysing such proteins, it is difficult to explain the relevance to POEMS syndrome pathogenesis based upon the molecules' individual function. However, STRING analysis of these proteins demonstrated a significant enrichment p-value, indicating the proteins do in fact have significantly more interactions than expected to occur by chance alone. Regulation of endothelial chemotaxis was linked by Fibroblast Growth Factor 2 (FGF2) and Heat Shock Protein 7c (HSP7c). Vascular endothelial growth factor signalling was linked by Neuropilin 1 (NRP1) and HSP7c, and complement activation was linked with Complement C3 (C3, C1 inhibitor (IC1) and Granulin Precursor (GRN). Such pathways appear in keeping with known POEMS pathogenesis, and some of the potential pathogenic pathways detailed in chapter 4, in particular the role of chemokines. Of the downregulated peptides, neuropilin 1 (NRP1) was -8.5 fold reduced compared to healthy controls. Neuropilin 1 is expressed on endothelial cells and acts as a co-receptor with VEGFR1 and VEGFR2 specifically for the $VEGF_{165}$ isoform which leads to pathogenic angiogenesis. [348] Neuropilin 1 could be considered a potential therapeutic target, in which blockade would prevent aberrant

angiogenic signalling by $VEGF_{165}$ whilst allowing the normal levels of physiological VEGF signalling to continue. It appears that Neuropilin 1 is therefore drastically downregulated in POEMS syndrome in order to diminish $VEGF_{165}$ signalling and pathological angiogenesis. Blockade of VEGF with Bevacizumab previously resulted in poor outcomes, possibly due to switching off important physiological functions. [29,200] Seeing that Neuropilin 1 is physiologically downregulated in disease indicates the potential importance of this receptor and suggests that pharmacological blockade may be of benefit as a treatment option. On the other hand, despite an 8.5 fold reduction of Neuropilin 1 in POEMS syndrome, $VEGF_{165}$ clearly continues to cause significant disease, and as such, blockade of Neuropilin 1 alone may not produce sufficient physiological effect upon vessel leak and angiogenesis in POEMS syndrome.

5.4.2.2 Proteins significantly altered compared to disease controls

Examining the proteins which were significantly altered to a similar degree to disease controls may assist in delineating the functional or pathological relevance of that protein. ICAM1, as noted previously, was elevated in both POEMS syndrome and CIDP, which may reflect the breakdown of the blood nerve barrier as mentioned before. Neurofilament medium (NfM) was markedly abnormal in both POEMS and CIDP, although, surprisingly, Neurofilament light and heavy were not. NfM levels were greater in CIDP than in POEMS syndrome but did not significantly differ between the two. NfM has been demonstrated before to accurately measure axonal loss in CNS pathology, [349] although NfL is a much more established biomarker of disease in the CNS and the PNS. We have shown in chapter 3 that NfL is raised in POEMS sera, and thus the lack of NfL

being identified as raised compared to controls in this study was surprising, but may reflect a lower sensitivity of this technique in detecting low abundance proteins. Tubulin A4A (TUBA4A) may also be of neuronal relevance, and was again raised in POEMS and CIDP. Tubulin forms microtubules, which are one of the three filament proteins which compose the cellular cytoskeleton of peripheral nerves. The release of tubulin may therefore be due to underlying axonal loss such as for NFM, however in this circumstance, TUBA4A levels were slightly greater in POEMS syndrome than CIDP (not significant).

Of the proteins shared between POEMS syndrome and myeloma, immunoglobulin heavy constant epsilon peptide (IGHE) was significantly reduced compared to CIDP and healthy controls, which may be related to the restricted immunoglobulin production typified by each condition. Heat shock proteins HSPs have been implicated in disorders of the PNS, particularly in gene mutations causing inherited neuropathies. The diversity of interactions and cytoprotective functions of HSPs make identifying the underlying role of individual HSPs in specific neuromuscular disease difficult. [350] Phospholipase D3 (PLD3) was highly elevated in POEMS syndrome and significantly reduced in myeloma, and this may have utility as a biomarker to differentiate one from the other. PLD3 is a neuronal lysosomal phospholipase which has been previously shown to correlate with levels of β-amyloid plaque density when studied in Alzheimer's disease. [351] This may be therefore associated with the raised amyloid precursor protein and abnormal protein misfolding, although the finding of amyloid deposition is not typical to POEMS syndrome, and if anything a more likely consequence of myeloma in which levels are in fact reduced.

5.4.2.3 Treatment effects

An alternative method to determine the relevance of a biomarker in disease is studying whether such is suppressed following effective therapy. Of the eight peptides which were found to be significantly and uniquely altered in POEMS syndrome compared to healthy controls, only three were significantly downregulated following treatment; heat shock protein 7c ($p=0.02$), cyclin-dependent-kinase-like 5 ($p<0.0001$) and cytochrome c ($p<0.0001$). Heat shock protein 7c is a chaperone protein with several reported roles, notably ensuring the correct folding of proteins and the targeting and clearance of misfolded proteins, [352] but is also considered to be involved in binding to bacterial lipopolysaccharide (LPS) mediating the inflammatory response involving the release of TNF. [353] Cyclin-dependent-kinase-like 5 is thought to be involved in neuronal function, since CDKL5 deficiency disorder, resulting from a genetic mutation in CDKL5 results in infantile epilepsy and neurodevelopmental delay. CDKL5 is a kinase, thus affecting the activity of target peptides. Ser-293 of amphipysin 1 (Amph1), a protein involved in clathrin-mediated endocytosis is phosphorylated by CDKL5 which is thought to suppress endocytosis. This plays an important role in several neuronal functions such as axon growth and synaptic vesicle recycling. [354] Cytochrome c-1 is a haem protein which is involved in the electron transport chain of mitochondria, and is often therefore detected in relation to cell apoptosis. It has been therefore identified in haematological malignancies as a marker of apoptosis during tumorigenesis. [355] Determining whether these proteins have a significant role in POEMS syndrome pathogenesis is unclear. The proteins themselves do not appear to be functionally related and are involved in different disease pathways. In addition, such peptides have not been identified

previously in CIDP, POEMS or myeloma through literature searching, suggesting they are either novel or potentially not relevant.

5.4.3 Limitations

Significant efforts are made to enrich low-abundance proteins from the complex serum matrix for mass spectrometry analysis, for example in this study by using the top ten depletion kit, and chromatographic seperation using acetonitrile. The top ten abundant proteins represent approximately 98% of the total protein content and thus need to be removed prior to study.[356] Immunoglobulins are removed as part of this, meaning the identification of immunoglobulins in this study were likely to be either enriched immunoglobulins that had been inadequately removed during sample preparation, or alternatively incorrectly identified proteins. Chromatography steps used to enrich low abundance proteins often also enrich high abundance proteins concurrently. Certain enriched proteins may represent 50% of the protein content in a particular fraction, suppressing the signals of the low abundance proteins.[356] Affinity chromatography columns can be used with specific antibodies to remove such high abundance proteins, such as heat shock proteins, but is not practical for every significantly abundant protein. Despite enrichment, the vast majority of proteins identified were seemingly insignificant proteins, irrelevant to the underlying disease processes being studied. Such proteins are likely detected purely on their abundance in the sample matrix, and may fluctuate as acute phase response molecules, or represent non-specific contaminants like auto-digest peptides, keratins, fatty acids, nucleic acids and artifacts. Such abundant proteins are likely to be detected across mass spectrometry experiments with similar methodologies, giving a false impression the protein is relevant. Peripheral nerve

specific proteins in particular are in low abundance and thus even large increases in the serum are likely to be comparatively low compared to that of proteins involved in other biological processes such as lipid metabolism or inflammation, for example. For this reason, trying to study the complete proteomic profile from the complex matrix that is serum to understand the entirety of pathological processes which underpin POEMS syndrome is unlikely to yield accurate results. The vast diversity of protein molecular weights, charges, hydrophobicity, conformational states, post-translational modifications and cellular distribution makes it unfeasible to use a single sample preparation protocol that sufficiently captures the entire proteome for a given biological system. Lastly, no single protein database is sufficient in characterising all useful peptide mass fingerprint spectra, and often spectra are subjected to different protein databases to characterise all spectra recorded. Concerningly, certain hypothetical proteins found in one database may be known, well defined proteins in another, demonstrating the limitations of database analysis of peptides. False positive identifications occur because large proteins deliver theoretical peptides with masses close to the measured and this may result in a random identity assignment. [356]

Inaccurate quantification of peptide through MRM-MS has many sources. Identifying proteins by detecting only a few fragment ions from a complex matrix is highly susceptible to misidentification and inaccurate quantification. [357] The key methodological limitation influencing such accuracy is related to interference and ion suppression by the components of the sample matrix. False positive identifications can occur when coeluting sample constituents also produce the product ions (or peptides with similar length amino acid residues) that elute very close in time and fragment

simultaneously with the analyte of interest. Suppression manifests as a decrease in the ion response for the same amount of analyte analysed from different samples, which increases with the complexity of the matrix. Even in circumstances whereby considerable efforts are made to remove non-volatile salts and to prepare samples as identically as possible, variations in sample constituents or chromatographic elution can produce interference or suppression affecting the results. In MRM-based mass spec, when tryptic digests of plasma are analysed, tens to hundreds of different peptides elute to the MS system simultaneously, meaning that isotope peaks of non-targeted peptides and their product ions can 'leak' into the selection windows, again producing false positive results. [358] Inaccurate quantification can also arise from other sources of variation, including unstable electrospray, poor chromatography and chromatographic peaks that are too narrow. Each of these factors compound the substantial inconsistency to the quantification of the peptide analyte, producing poor reproducibility and high CVs that reduce overall accuracy and quantification.

The seemingly unrelated spectrum of proteins identified through the targeted study was likely a result of the methodology adopted, reflecting the fact that although the panel of peptides selected was broad, it remains selective and thus only detected the proteins it was assigned to measure. This means that potentially relevant or more disease specific proteins may have been overlooked because the panel was not representative of the relevant spectra of disease. This limitation can be overcome by designing a disease specific targeted multiplex assay, selecting biomarkers and related peptides either previously identified through alternative methodologies, or through an untargeted mass spectrometry study. In addition, analysis of mass spectrometry data is

complex and requires manual inspection of internal standard chromatographic peaks and consistent selection of corresponding peaks from endogenous samples. Therefore the potential for false positive peak picking and subsequent inaccurate quantification of peptides in highly complex samples is higher than when performing the immunoassays discussed in previous chapters.

5.4.4 How do these data advance our knowledge of POEMS syndrome pathogenesis?

To conclude, this chapter provides evidence of POEMS syndrome as a haematological malignancy which switches on a proinflammatory state, resulting in activation of immunity including cytokines, chemokines and the complement pathway, expression of immunoglobulins, lipid metabolism, blood nerve barrier breakdown and clotting abnormalities. Inflammation by LXR/RXR activation, acute phase response and complement activation were key pathological processes detected through untargeted serological study. This study has identified lipid metabolism as a potentially relevant pathological process in POEMS syndrome not previously recognised. Myeloma research has demonstrated the significance of the bone marrow microenvironment, rich in immune cells, bone cells, mesenchymal stroma, growth factors (including VEGF), and cytokines (including IL-6) which support the plasma cell differentiation, proliferation, survival and drug resistance. [334] ApoE appears to be produced in response to systemic inflammation to mediate the immune system and support neuronal regeneration. Serum amyloid proteins likely result from systemic activation but may also lead to further provocation of the immune system. The humoral immune system dysregulation, through production of an aberrant plasma cell clone, immunoglobulin production and

complement activation are potentially significant steps in POEMS syndrome pathogenesis.

Mass spectromic analysis demonstrated abnormal immunoglobulin heavy and light chain markers which differed to the previously identified restricted IGLV 1-40 and IGLV 1-44 germline sequences. The fundamental complexities in antibody composition and limitations of mass spectrometry may have resulted in incorrect immunoglobulin compositions being detected. Generating IgG variable gene sequences through NextGen sequencing of mature aberrant B-cell clones in POEMS syndrome may enable more accurate identification from subsequent serological samples.

Complement pathway activation is likely a result of immunoglobulin production and antigen presentation, but the understanding of how interactions occur and the downstream effects such as active complement component metabolites and MAC production require further study.

Neuropilin-1 is significantly downregulated, presumably in an attempt to reduce the pathological effects of VEGF isoform A whilst allowing the necessary physiological functions of other VEGF isoforms to continue. Hypercoagulation resulting in arterial and venous thrombosis have been demonstrated as significant features of POEMS syndrome, as detailed in chapter 2. Several anti-coagulation markers were identified in this study, which are potentially confounded by heparin treatment as opposed to underlying disease, or through using serum samples rather than uncoagulated plasma and thus

appropriate samples need to be retested in patients prior to initiation of tinzaparin therapy.

The blood nerve barrier appears to be of significant physiological relevance in POEMS syndrome and that of other immune mediated neuropathies. There are clear data demonstrating the influence of ICAM1 on blood brain barrier permeability, and it is therefore possible similar processes occur at blood nerve barrier. ICAM1 is significantly elevated in both POEMS syndrome and CIDP but not myeloma, which may account for neuronal damage in the former two conditions. Inhibition of ICAM1 may therefore maintain blood nerve barrier integrity, allowing persistence of immune privilege, thus protecting the peripheral nerves from otherwise toxic damage.

Neuropathy markers were not identified in the untargeted mass spec study, which is likely due to the relatively low abundance of such biomarkers in the serum. Cofilin-1 may be generated in response to neuropathy to support regeneration, in combination with apoE. Despite neurofilament light being mostly studied as a biomarker of neuronal injury, targeted measurement revealed the release of neurofilament medium and tubulin A4 in both POEMS and CIDP reflecting underlying axonal loss.

To develop this work further, the untargeted study should be repeated using a reference library curated by NextGen sequencing of mature aberrant B cell clones in POEMS syndrome in order to produce a reference library containing the full antibody repertoire of POEMS syndrome to compare against for subsequent analyses. In doing so, this would

improve the accuracy of immunoglobulin detection and characterisation. Secondly, data gathered primarily from the shotgun proteomic study, but also potentially relevant markers picked up from the targeted study should be amalgamated with data from the literature to specify the key peptides involved in POEMS syndrome. Using such data, a POEMS syndrome specific targeted multiplex panel should be created to identify, quantify and compare the peptide fingerprint of POEMS syndrome in pre- and post-treated disease, and following relapse rather than using a preconfigured panel for a different condition. Samples from the UK-POEMS cohort could be analysed first, then confirmed with a validation cohort from overseas collaborators. Development of such a panel may provide a broader overview of the multiple independent but interlinked functional pathways occurring in POEMS syndrome concurrently. This would include immunoglobulin subtypes, inflammatory proteins including cytokines, chemokines and complement, neuronal markers including the neurofilaments, tubulin A4, cofilin1 and ICAM1, coagulation and endocrine markers. Such a panel would provide detailed quantitative measurements reflecting the various features of disease either present or at risk. Stratifying patient groups dependent on clinical findings, prognostic features or treatment response may further enrich the data gathered.

6. *In vitro* and ex vivo modelling of peripheral nerve pathology in POEMS syndrome

6.1 Introduction

Previous chapters in this thesis have uncovered a number of underlying pathological mechanisms of POEMS syndrome which suggest the production of a toxic milieu within the systemic circulation, which likely has a number of downstream effects upon target organs leading to multi-system disease. The purpose of this chapter was to study whether such toxic, pathologic effects could also be demonstrated at the level of the peripheral nerve. In doing so, this would possibly explain why neuropathy occurs as a characteristic and specific feature of POEMS syndrome, and potentially identify key pathogenic mediators of neuropathy, which may have important therapeutic applications.

In order to study neuronal pathology in POEMS syndrome, I collaborated with my secondary supervisor, Dr Simon Rinaldi, at the Nuffield Department of Clinical Neurosciences whose laboratory specialises in cell-based models of immune mediated neuronal injury, in particular using human induced pluripotent stem cells. Preliminary data suggested there may be a role of the blood nerve barrier in POEMS neuropathy, and we therefore additionally collaborated with Professor Alison Llyod at the UCL MRC Laboratory for Molecular Cell Biology, whose laboratory had created powerful *in vivo* and *in vitro* models to understand more about peripheral nerve structure and function, particularly that of the blood nerve barrier. Utilising both systems would enable me to study the neuropathological processes which take place in POEMS syndrome.

The following chapter provides an introduction to the experimental techniques that were to be utilised to study pathology of the peripheral nerves in POEMS syndrome. Clark *et al* developed and optimised a human induced pluripotent stem cell-derived sensory neuron model in Oxford to study peripheral nerve pathogenesis. [359] This technique has been mostly utilised thus far to identify antibody binding to antigenic peripheral nerve targets, such as anti-disialosyl or nodo-paranodopathy antibodies. These methods are in many ways novel, somewhat in their infancy and have not previously been applied to study POEMS or similar mechanisms, but are likely to inform our knowledge of peripheral nerve biology. Although COVID-19 has severely disrupted the final stages of this research project, a combination of important clinical and pathological discoveries relating to POEMS syndrome have been made, and further directions of research identified.

The purpose of this study was therefore to;

1. Provide proof of concept that non-antibody mediated peripheral nerve damage could be demonstrated *in vitro*, and
2. that patterns of peripheral nerve damage observed *in vitro* would reflect pathological processes that occur *in vivo*.

Demonstrating peripheral nerve damage occurring following exposure to disease serum constituents and not relevant controls would provide some evidence to support the hypothesis that soluble, non-antibody mediators (such as cytokines) can directly damage peripheral nerves. If these pilot data were suggestive, then further, more

detailed experimentation could be conducted looking at exposing neuronal cultures to specific serum components, to identify the particular neuropathy-causing mediators. This may also enable further work to consider how blocking such compounds could prevent nerve damage in POEMS syndrome.

6.1.1 Existing models of peripheral nerve injury and repair

The histopathology of *ex vivo* human peripheral nerves is very informative, but this is only accessible from pathological tissue biopsies during life, or from autopsy. *In vitro*, *in vivo* animal and *ex vivo* models of peripheral nerve injury allow for higher throughput experimentation in controlled conditions, enabling repeated or continuous study over time. Although not an exhaustive list, the section below details key models of nerve injury and repair which have provided significant developments to our understanding of acquired peripheral nerve disease.

6.1.1.1 Experimental Autoimmune Neuritis

Experimental autoimmune neuritis (EAN) is an acute inflammatory demyelinating polyradiculopathy that can be induced in rats, mice, rabbits and monkeys by active immunisation with whole peripheral nerve homogenate, myelin and myelin proteins P0 and/or P2, or peptides thereof. Over time, more autoantigens have been identified to induce EAN, such as myelin basic protein (MBP) peripheral myelin protein-22 (PMP22) and myelin associated glycoprotein (MAG). [360] The pathological hallmark of EAN is the infiltration of the PNS by lymphocytes and macrophages which results in multifocal demyelination of axons predominantly around venules. EAN is felt to reflect the

immunological, electrophysiological and morphological aspects of human Guillain-Barré syndrome (GBS). The study of EAN has developed our understanding of key pathological steps that take place in GBS: blood nerve barrier breakdown, autoreactive T-cell activation and migration to the PNS, engagement with macrophages, clonal T-cell expansion and cytokine (notably TNF-α and interferon-γ) release. [361,362]

6.1.1.2 Experimental disease with glycolipids

Immunisation of rabbits with glycolipids can also induce an inflammatory neuropathy. Glycosphigolipids have a common but diverse structure, with an intramembrane ceramide and extracellular sugar moiety. Galactocerebroside (Gal-Cer) is the simplest of these and sulfatides and gangliosides represent molecules of increasing complexity.

Immunisation with galactocerebroside, the principal glycolipid antigen of peripheral and CNS myelin, induces a demyelinating neuropathy. [363] Anti-galactocerebroside antibodies can also induce demyelination following intraneural injection. [363]

There are more than 100 ganglioside species in humans, present in all tissues but particularly abundant in the nervous system. Immunisation of rabbits with a mixture of bovine brain gangliosides or isolated GM1 led to an acute monophasic flaccid limb weakness.[364] The nerve histopathology demonstrated predominant axonal involvement with Wallerian degeneration, little lymphocyte infiltration, and only minimal demyelination, and thus was thought to represent a model closely related to that of

acute motor axonal neuropathy. These difficult models are thought to be a surrogate for the molecular pathogenesis of axonal GBS.

Similarly, studies have also demonstrated that the injection of IgM anti-GM1 antibodies from patients with MMN into rat tibial nerves induced conduction abnormalities. [365] A study by Arasaki *et al* (1993) also demonstrated conduction block in a novel *ex vivo* system using rat sciatic nerve in a conduction chamber exposed to serum whilst simultaneously recording neurophysiological data. [366]

IgM antibodies against GD1b are often detected in chronic sensory ataxic neuropathies including CANOMAD syndrome. An experimental sensory neuropathy model has also been induced by sensitisation with purified GD1b, through hindfoot and intradermal injections in mice. This results in a brisk production of GD1b antibodies, ataxia, axonal degeneration in the dorsal roots and preservation of sciatic motor conduction parameters. [367]

6.1.1.3 Chronic dysimmune neuropathy model

The pathogenic role of anti-MAG antibodies was demonstrated by intraneural injection of sera from patients with anti-MAG neuropathy into feline or rabbit sciatic nerves, or by passive transfer into chicks. Characteristic segmental demyelination, myelin splitting and IgM deposits were seen on the outer myelin lamellae, similar to that observed in human anti-MAG neuropathy.[368,369] Although a useful model to study the

neuropathological effects of anti-MAG antibodies, the animals in the studies did not exhibit a clinical phenotype of anti-MAG neuropathy, which reinforces the critique of animal models in that they do not always represent the same disease in humans.

6.1.1.4 Phrenic nerve-diaphragm preparation

Ex vivo studies have been performed with mouse nerve-diaphragm preparations, from which miniature end plate potentials (MEPPs) can be recorded following stimulation of the phrenic nerve via a ring electrode. [370] This motor nerve – muscle preparation has been used to investigate the pathogenicity of antibodies in inflammatory, immune mediated neuropathies at the presynaptic component of the neuromuscular junction, another vulnerable site for antiganglioside injury.

Roberts *et al* (1995) demonstrated that sera from patients with MMN can interfere with distal motor nerve function in the mouse phrenic nerve hemidiaphragm preparation, whether studied after direct application of sera to the model, or following *in vivo* injection of sera in mice followed by tissue harvesting and *ex vivo* testing.[370] The same group have also demonstrated similar results from anti-GQ1b positive Miller Fisher serum compared to patients with other neurological diseases and healthy controls.[371]

6.1.1.5 Mechanical nerve injury

Nerve injury, pain and regeneration rely on animal models for experimentation. Several nerve injury methods exist, such as nerve transection, chronic constriction, partial sciatic nerve ligation and spinal nerve ligation, each of which provide different durations and magnitudes of evoked response. These models result in reproducible sensory changes (allodynia, hyperalgesia and spontaneous pain) measured with hot and cold plates, Von Frey probes, pinpricks, and cold acetone[372] Behavioural observations such as the sciatic functional index, pinch reflex test, walking track analysis and grooming tests provide disability outputs.[373]

6.1.1.6 Nerve-on-a-chip

Tissue chips are engineered microsystems that represent units of human organs, such as the lung, liver or heart, modelling both structure and function. Nerve-on-a-chip is a novel *in vitro* biophysiologcal system for mimicking peripheral nerve physiology. This technology works by lining clear, flexible polymer 'chips' containing microfluidic channels with induced pluripotent stem cell derived neurones and culturing these to produce a microengineered peripheral nerve structure. This model can theoretically be exposed to drugs or potential pathogenic substrates, and both neurophysiological and histopathological assessment can be performed. The authors propose that this is the first *in vitro* system which has managed to use human Schwann cells to myelinate human neuronal cells, which they attribute to their formation of a 3D spheroid comprised of myelinated with primary human Schwann cells. In simple terms, human Schwann cells and neurones are first cultured to form a spheroid, and placed into the 3D

manifold, which then enables combined neurone growth and myelination of axons in selected media.[374].

6.1.1.7 *In vitro* stem cell-based models

Although *in vivo* animal models may represent human peripheral nerve circuitry, they cannot necessarily reproduce neurobiological events occurring in humans, raising the question of whether animal models are reliable surrogates for modelling human diseases. *In vitro* studies provide an attractive alternative since an unlimited source of biological material can be generated using differentiated cells which display several hallmarks of *bona fide* mature peripheral neurones.

The derivation of peripheral sensory neurones from human induced pluripotent or embryonic stem cells is well described. [375–377] Some systems have used both human derived neuronal *and* Schwann cells, but none have been successful in developing structural myelin segments and other axoglial landmarks such as nodes of Ranvier. The co-culture of human induced pluripotent stem cell derived sensory neurones with myelinating rat Schwann cells is a hybrid system developed by Clark and Rinaldi *et al* which produces more structurally representative neuronal cultures, albeit not fully humanised. [359] They have demonstrated these co-cultures have the expected markers of specialised axoglial relationships including nodes of Ranvier. HiPSC derived systems are also stable and survival is in the order of months rather than the weeks of primary cultures.

Myelinated co-cultures provide significant benefits to simpler neuronal cultures, as dysimmune processes underlying inflammatory neuropathies target distinct topographical locations of the peripheral nerve including the myelin sheath and the node of Ranvier. [378] Although the authors have only demonstrated antibody mediated pathology in this co-culture system, their stability is also of benefit to non-antibody mediated pathologies, such as cytokine or drug-toxicity effects requiring long term exposure.

6.1.1.8 *In vitro* modelling of the blood nerve barrier

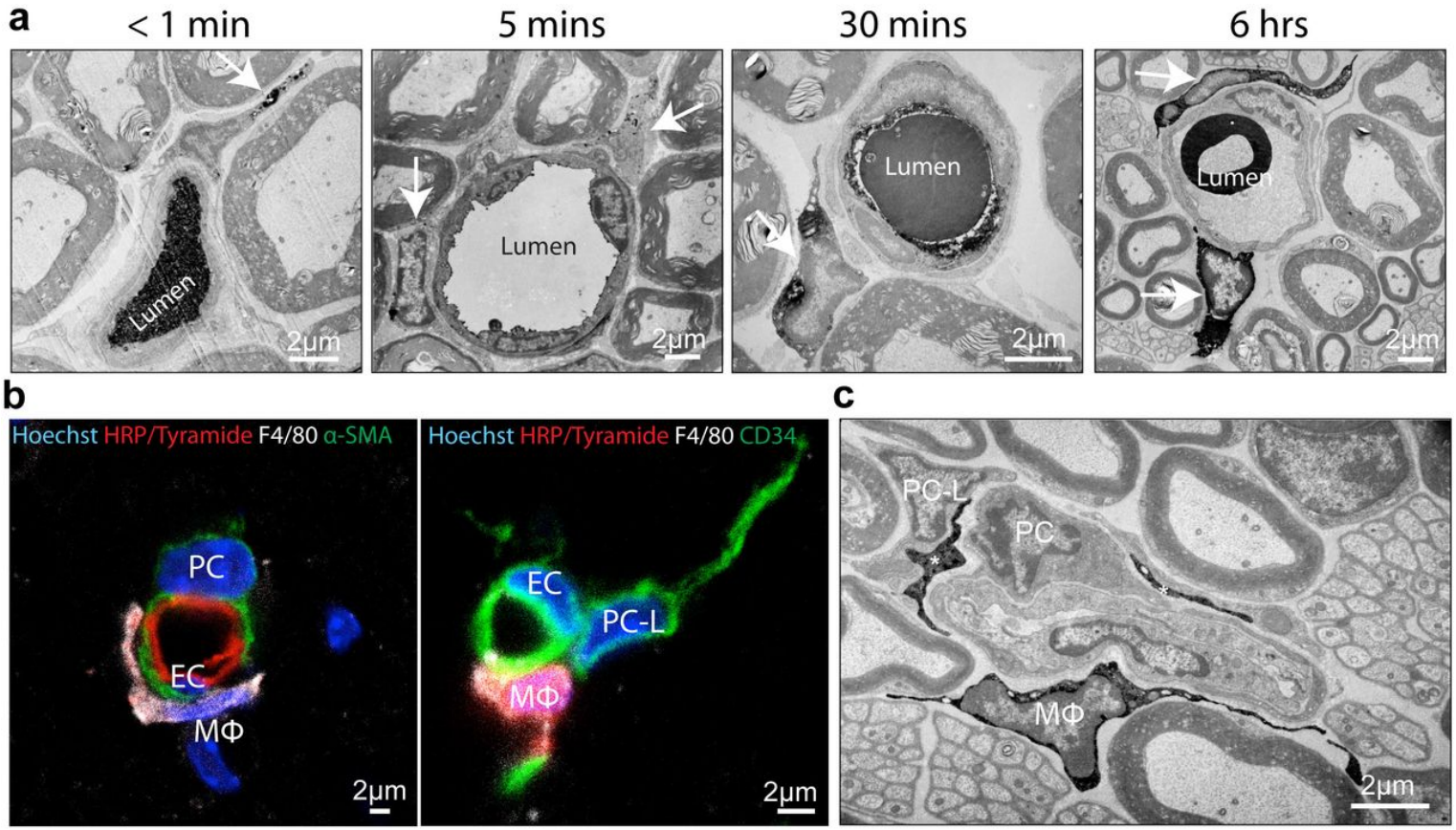
Breakdown of the blood nerve barrier is a key pathogenic step of most, if not all inflammatory neuropathies. To study the cellular properties of the peripheral nerve microvascular endothelial cells (PnMECs) of the BNB, Kanda *et al* have established an immortalised cell line derived from human PnMECs.[379] These cells exhibit similar morphology and protein expression as those *in vivo*. In addition, transendothelial electrical resistance and permeability studies can examine functional barrier properties in response to different environmental stressors including those in disease. This model has been subsequently utilised to examine the effect of GBS, CIDP, MADSAM and MMN sera on the BNB,[90] cytokine production response to damage,[380] the influence of anti-GM1 antibodies and leukocyte trafficking. [381]

6.1.1.9 Animal modelling of the blood nerve barrier

The UCL Medical Research Council Laboratory for Molecular Cell Biology utilise advanced imaging technologies and molecular analyses to determine both the structural

composition and function of the blood nerve barrier in mice. They have optimised experiments to assess the barrier function of the BNB through injecting mice with HRP and Evans Blue, dissecting and then imaging of peripheral nerves in 3D with electron microscopy.[382] They have demonstrated that macrophages in close proximity to the BNB are responsible for engulfing materials that penetrate the BNB. We felt this model could be exploited to examine the effects of particular cytokines, notably VEGF on permeability and barrier function of the BNB.

Figure 6.1: Electron microscopic imaging demonstrating blood nerve barrier permeability and macrophage function



A= Animals injected with HRP and harvested immediately, at 5 mins, 30 mins and at 6 hours. Arrows display perivascular macrophages ingulfing HRP. B= Fluorescent confocal imaging showing macrophages (white) take up HRP (red) whereas pericytes and pericyte like cells (green) do not. C= demonstrating macrophages (MΦ) accumulate HRP compared to other BNB cells (PC, PC-L) which do not. Taken from Malong et al 2019.[382]

6.1.2 Hypotheses

1. POEMS syndrome sera causes damage to nerves by both demyelination and axonal loss demonstrable in HiPSC derived myelinated neuronal co-cultures.
2. Similar diseases without peripheral neuropathy do not cause damage to neuronal cultures.
3. Progression of neuronal damage can be captured by confocal microscopic imaging.
4. Neuropathy is cytokine mediated, independent of antibodies
5. Specific cytokines cause neuronal damage in myelinated neuronal co-cultures.
6. Disruption of the blood nerve barrier by VEGF may be a key pathogenic event in peripheral neuropathy in POEMS syndrome.

6.1.3 Aims

1. Expose myelinated neuronal co-cultures to serum from patients with monoclonal plasma cell diseases with and without neuropathy, and other related inflammatory neuropathies.
2. Image cultures across time points to identify progressive damage
3. Separate antibodies from sera and test both antibody and remaining serum effects on neuronal co-cultures.
4. Test individual cytokines (VEGF, IL-6, IL-16, IL-7) on neuronal co-cultures at varied concentrations to ascertain whether damage can be identified.
5. Demonstrate that high circulating VEGF leads to increased permeability of the BNB through a mouse model of the BNB

6.2 Modelling peripheral nerve damage in POEMS syndrome using a HiPSC methodology

6.2.1 Methods

6.2.1.1 Patient samples

Patient disease and control samples were used to investigate whether expected pathological changes to the neuronal co-cultures could be demonstrated. Two cases with pre-treated POEMS syndrome, two cases of Castleman Disease POEMS syndrome (without clinical evidence of neuropathy), two CIDP, two multiple myeloma and two multi-focal motor neuropathy cases were selected. Each case was selected as satisfying typical disease diagnostic criteria, having severe evidence of disease and samples being prior to initiation of therapy. Positive control samples selected were human serum from a Miller Fisher-GBS overlap, and human serum from a patient with Zika virus induced GBS, which have previously been demonstrated to cause degeneration, or demyelination, respectively using the co-culture system. Negative controls of normal human sera, and culture media only, were also used.

6.2.1.2 iPSC reprogramming

Cell line AD2-1 (from a 51-year-old male) was reprogrammed using the CytoTune-IPS Reprogramming Kit (ThermoFisher). Fibroblasts to generate AD2-1 were obtained from a commercial source (Lonza,CC-2511). The iPSC line AD2-1 was obtained through the IMI/EU sponsored StemBANCC consortium via the Human Biomaterials Resource Centre, University of Birmingham, UK (http://www.birmingham.ac.uk/facilities/hbrc).

iPSC lines were also subject to strict quality control checks before the initiation of differentiation. Quality control details for AD2-1 are explained in Van de Bunt 2016. [383]

The CytoTune reprogramming kit contains four Sendai virus based vectors each capable of producing one of the four Yamanaka factors (KLF4, OCT3/4, SOX2 and c-MYC) which if overexpressed can induce pluripotency. Cells were then cultured for 5-6 days with alternate day medium changes. Transduced fibroblasts were then passaged onto pre-prepared feeder layer plates using mitotically inactivated mouse embryonic fibroblasts (MEF). Three to four weeks following, colonies should have grown to appropriate size to allow for manual picking. Using an inverted microscope, a single colony displaying iPSC morphology was cut into 5-6 pieces using a 25G needle, transferred to iPS media and plated onto MEF plates. Colonies were allowed to attach for 48 hours, and thereafter medium changes were performed daily. iPSCs were adapted to feeder-free conditions onto Matrigel coated plates in mTeSR1 medium. Bulk passaging was by 0.5 EDTA to make large scale stocks cryopreserved in liquid nitrogen.

6.2.1.3 Induced pluripotent stem cell maintenance and differentiation

iPSCs were defrosted, plated onto Matrigel® coated plastic and maintained in mTeSR1 medium, which was exchanged daily. iPSCs were passaged when 90% confluent onto Matrigel® coated plasticware using TrypLE express (ThermoFisher). Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor (ScienCell) (10 μM) was included in the medium for 24 h after each passage. Prior to the start of differentiation, iPSCs

were plated onto Matrigel® coated 6-well plates. Twenty-four hours later, the medium was exchanged from mTeSR1 to mouse embryonic fibroblast conditioned medium (ScienCell) supplemented with 10 ng/ml human recombinant FGF2. Cells were allowed to expand in MEF-conditioned medium until ~50% confluent, at which time differentiation was started accordingly as according to the protocol detailed by Clark et al.[359] This process involves a gradual transition from knockout serum replacement medium to N2 medium over an 11 day period. On day 11 the immature neurones were replaced using TrypLE (Gibco) onto Matigel coated coverslips in 100% N2 medium containing human recombinant NGF, GDNF, BDNF and NT3. Medium changes were then performed twice weekly. This differentiation resulted in a pure neuronal culture with extensive arborised neurites by 2–3 weeks after the end of the small inhibitor stage.

6.2.1.4 Schwann cell harvesting and culture

All work using animals conformed to UK Home Office legislation (Scientific Procedures Act 1986). Schwann cells were harvested from the sciatic nerve and brachial plexus of postnatal day 3 or 4 rats. Rat Schwann cells are used as established culture protocols exist and they can be successfully expanded several times *in vitro* to create cryopreserved stocks. Nerves were enzymatically digested with a collagenase (Sigma, 3 mg/ml) and dispase II protease (Sigma, 2.5 mg/ml) incubation for 1 h at 37°C. Nerves were gently triturated using a P1000 pipette tip and plated onto PDL/laminin coated plastic in Schwann cell expansion medium [DMEM/F12 (ThermoFisher), 10% foetal bovine serum (ThermoFisher), 200 ng/ml NRG1-β1 EGF domain (R&D Systems), 10 ng/ml NGF (recombinant-murine, Peprotech), 4 μg/ml forskolin (Sigma)]. Cells were

serially treated with 5–10 μM araC to eliminate fibroblasts. After approximately four passages, each time doubling the growing area, cells were frozen in a Mr Frosty freezing container (ThermoFisher), and stored in liquid nitrogen.

6.2.1.5 Establishing the myelinating co-cultures

To establish the co-cultures, a frozen vial of Schwann cells was thawed in warm water, centrifuged in phosphate-buffered saline (PBS) and resuspended in Schwann cell basal medium [DMEM/F12 (ThermoFisher), 5 μg/ml insulin (Sigma), 100 μg/ml transferrin (Millipore), 25 ng/ml NGF (recombinant-human, Peprotech), 25 ng/ml Selenium (Sigma), 25 ng/ml thyroxine (Sigma), 30 ng/ml progesterone (Sigma), 25 ng/ml triiodothyronine (Sigma), 8 μg/ml putrescine (Sigma)]. The medium on the neuronal cultures was changed from N2 to Schwann cell basal medium. Schwann cells (25 000) in 100ul solution was pipetted into each well of a 96 well plate containing the iPSC-derived neurons, with care taken not to touch the base with the pipette tip. Cultures were carefully put back into the incubator to allow Schwann cell adherence. The culture medium was changed 2 days later. An additional Schwann cell basal medium change was performed 4 days after adding the Schwann cells. Myelination was induced 1 week after Schwann cell addition, by exposing the cells to myelination medium [N2 medium, 1:300 phenol-free Matrigel® (Scientific Laboratory Supplies), 5% charcoal-stripped FBS (ThermoFisher), 25 ng/ml NGF (recombinant-human, Peprotech), 50 μg/ml ascorbic acid (Sigma)]. Medium changes were performed twice weekly from then on.

6.2.1.6 Incubation with patient sera

Firstly, fluoromyelin red fluorescent myelin stain (Thermofisher) was added at 1:300 to N2 and NGF media, and 100ul was added to each well of the 96 well plate, and incubated for 1 hour at room temperature. Media were then replaced using N2 complete and NGF only, and imaged using confocal microscopy for baseline images. Serum samples were then diluted 1:5, 1:50 and 1:100 using a 1:400 human NGS and 1:300 matrigel. 100ul of the serum dilutions were added as quadruplicates to wells of the 96 well plate and left overnight incubating at 37^0C. After 24 hours, wells were washed with N2 complete and restained with the fluoromyelin media, incubated for one hour and re-imaged. Following imaging, the same (but fresh) serum dilutions were added back to the wells for further incubation for one week before re-imaging, washing and further serum added, and finally imaged at 2 weeks.

Due to considerable cell death in the first experiment (including under control conditions), the methodology was refined to starting the experiment by adding serum at 1:5, 1:50 and 1:100 to cultures in 1:400 human NGS and 1:300 matrigel and incubating for one week (with 2 media changes), before permeabilising, fixing and labelling. Cultures were permeabilised by adding ice cold methanol for 25 minutes, washed three times with PBS and blocked with 5% normal goat serum in PBS for one hour. Primary antibodies were made up including chicken anti-NF200 (Abcam) at 1:10,000 as an axonal stain, rat anti-MBP (Abcam) at 1:500 as a myelination stain and rabbit anti-AFT3 (Abcam) at 1:100 as a neuronal cell body stain. 100ul of each was added to each well, and incubated for one hour at room temperature. Wells were washed three times with DMEM/HEPES and anti-chicken-biotin (Abcam) at 1:200, anti-rat-alexa fluor546

(Abcam) 1:1000 and anti-rabbit-alexa fluor488 (Abcam) at 1:1000 were added at 100ul to each well, and incubated at room temperature for one hour before washed three times and tertiary antibody streptavidin-Pacific blue was added at 1:100 as 100ul to each well and incubated a further hour. Cultures were then washed three times with DMEM/HEPES before imaging performed using a confocal microscope.

6.2.1.7 Confocal imaging and quantification

A calibration point was set in well A1 of the 96 well plate to focus the confocal lens. Zen Black Software (Zeiss) was used to produce automated stage movement with defined coordinates to allow serial imaging of the precise location of each well upon subsequent imaging rounds. In addition, the previously recorded image was used as a background template to which the new image could be overlaid (using recognisable neuronal structures to overlay images) for small manual adjustments to ensure the image frame was the same and consistent across time. Images were created adjusting the threshold to acquire the maximum signal-to-noise ratio. When imaging fluorescent labelled antibody stains, images were created for each channel (MBP, NF200 and AFT3).

Images were processed using ImageJ software. Fluoromyelin stained neurones were measured by manual freehand tracing using a touch screen computer for accuracy, and combining all neuronal lengths recorded within the 640x640 um field. For fluorescent labelled antibody stains, ImageJ was used to measure both the NF200 positive surface area as a measurement of axon area, whilst MBP positive surface was used as a measurement of myelin area. When comparing between conditions, the proportion of

total axonal area covered by myelin (MBP area/NF200 area) was calculated, and expressed as a ratio compared to control.

6.2.1.8 Ethics

Human iPSC lines used in this study were derived from human skin biopsy fibroblast samples. Reprogramming of cell lines was granted approval from the research ethics committee; National Health Service, Health Research Authority, NRES Committee South Central, Berkshire, UK (REC 10/H0505/71).

6.2.2 Results

6.2.2.1 Neuronal co-cultures exposed to serum over time

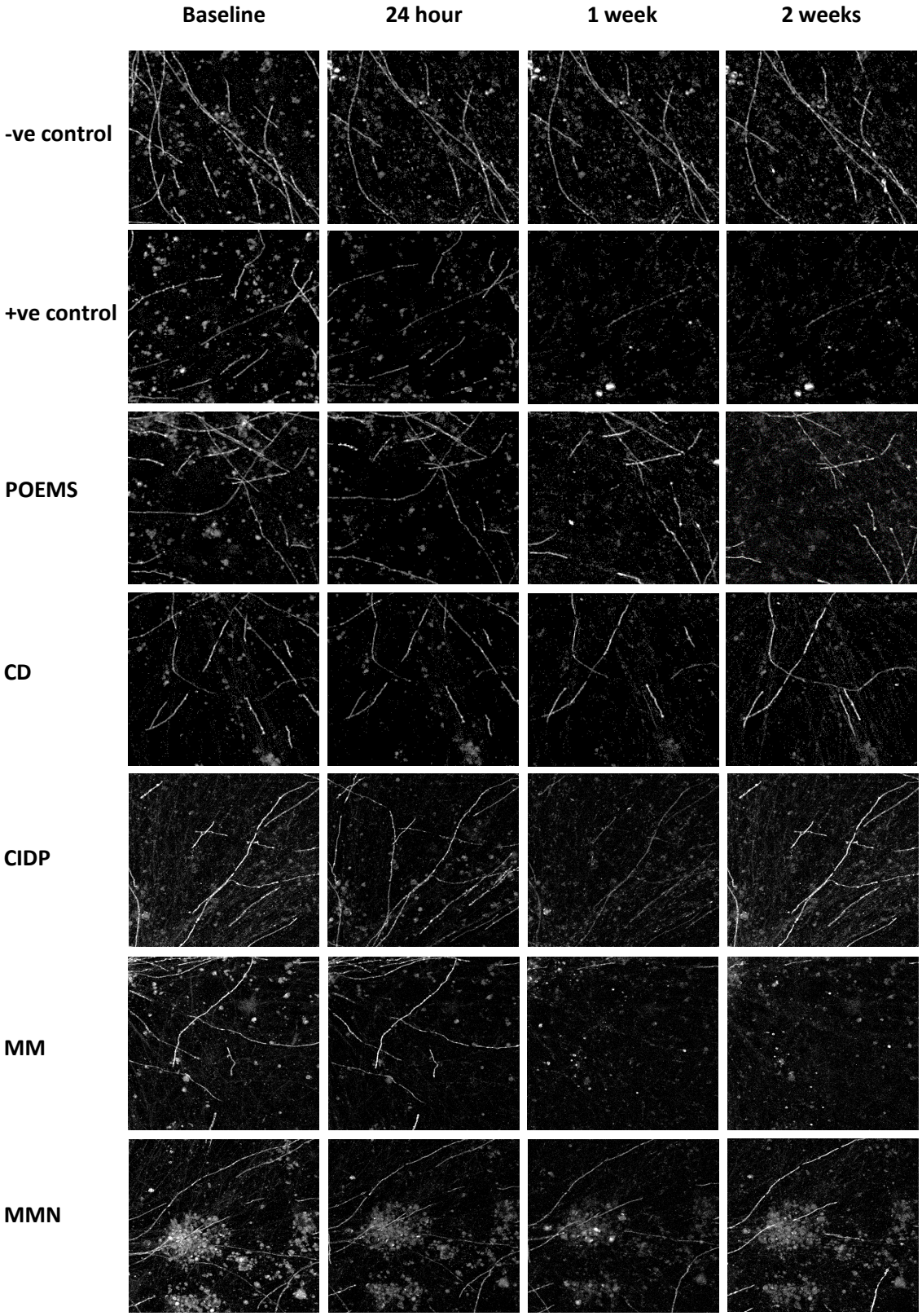
Initial imaging of neuronal co-cultures at baseline demonstrated successful axonal growth with concurrent myelination, as demonstrated through fluoromyelin staining (figure 6.2). Positive control samples (Zika and Fisher-GBS overlap antibody treated) displayed significant neuronal loss at 24 hours with almost 50% loss of myelinated axons, to close to complete absence of myelinated axons by one week. The negative control samples had much lower but significant levels of myelinated axon loss (approximately 31% over two weeks) thought to be due to degradation of samples over time, contributed to by the need to repeatedly remove the 96 well plates from the incubator to image the wells at the various timepoints. Imaging all wells successfully takes around 4 to 5 hours, in which time it is likely that exposure to room temperature affects the health of the culture, and may also expose cultures to airborne pathogens.

This background neuronal degradation influences interpretation, as the impact of disease serum has to be interpreted in the context of this background loss.

When studying neuronal co-cultures exposed to POEMS serum samples, the loss of myelinated neurones appeared to be slightly greater than that of the negative control samples (delta % change 31 vs 43%), but the significance of this is uncertain given the external factors and measurement error. The same applies for the other inflammatory neuropathies with similar delta % change of neuronal loss at two weeks.

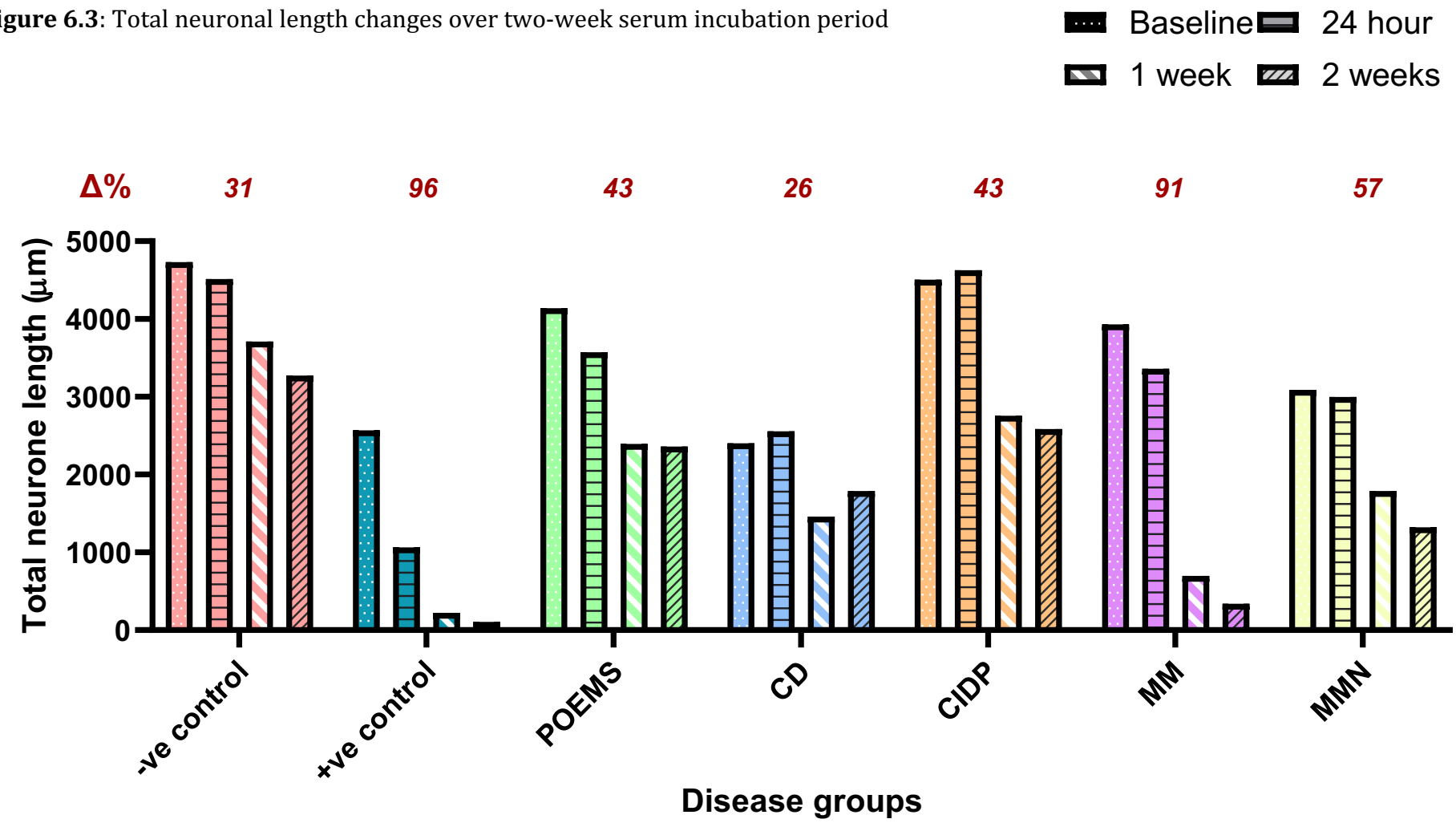
Interestingly multiple myeloma sera appeared to obliterate neuronal samples within the space of one week, to a similar degree to that of the positive control sample (see Figure 6.2). This also appeared to be concentration dependent, in that more pronounced neuronal loss was observed using serum diluted 1:5 with a 91% reduction in neurones compared to 59% at 1:50 and 39% at 1:100 (data not shown).

Figure 6.2: Confocal imaging of myelinated neuronal cultures stained with fluoromyelin



Neuronal co-cultures were imaged at baseline, then exposed to disease or control sera (labelled on left) diluted 1:5. Culture was re-imaged at 24 hours, 1 week and two weeks to determine whether nerve damage could be imaged over time.

Figure 6.3: Total neuronal length changes over two-week serum incubation period

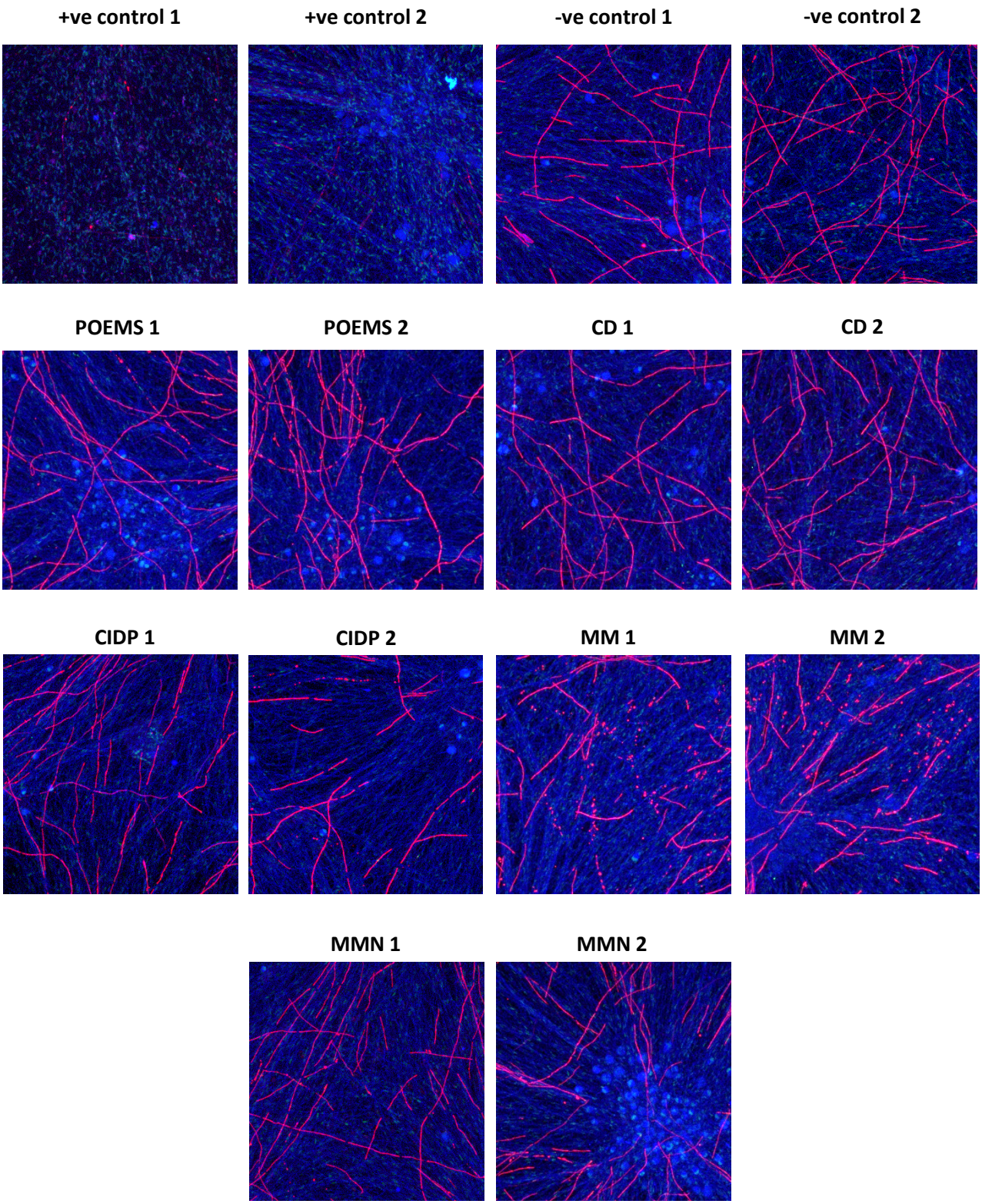


All samples exposed to serum at a 1:5 dilution of disease or control serum. Myelinated neurones were measured over a 640x640 um grid to calculate the total neuronal length at time points of baseline, 24 hours, 1 week and 2 weeks. Red number centred above bars labelled Δ% represents % delta change of total neuronal length from baseline to 2 weeks.

6.2.2.2 Neuronal cultures incubated for one week

Due to significant background neuronal loss during the experiment above, we next exposed the cultures to serum, and then incubated for a week with removal only for media changes before fixing and imaging. Example images from this experiment are displayed in figure 6.4. Overall, samples were very densely populated with neuronal axons (seen by the blue NF200 antibody). Positive control 1 caused almost complete axonal and myelin loss, and control 2 profound demyelination only. Because baseline images were not taken in this experimental set up, background neuronal degeneration (i.e. comparing baseline neuronal tissue area to that at one week) could not be calculated. However, comparing disease samples to controls, there was not any significant axonal loss or demyelination from the peripheral neuropathy disease groups (POEMS, CIDP, MMN) other than infrequent 'blebs' where some myelin damage had occurred. This was also apparent when comparing myelin area to axonal area against negative controls, where myelin: axonal areas were between 0.76 to 1.57 of controls. As before, myeloma samples, particularly at 1:5 dilution display significant disruption to myelin compared to controls and other disease groups (see figure 6.4). Myelin/axonal area compared to control samples was 0.63, with a significantly reduced intact myelinated axon length (mean 4850 μm average in control (N= 2), 5490 μm in POEMS syndrome (N =2), and 2452 μm for myeloma (N= 2).

Figure 6.4: Neuronal sample damage from one week incubation with disease sera



Cultures imaged using anti-NF200 antibodies for axons (in blue) and anti-MBP for myelin (red). Image frame 640x640 um field.

The cell death induced by MM samples was unexpected, since myeloma does not usually cause neuropathy, and the neuropathy causing conditions, in particular POEMS syndrome, did not result in neuronal damage in this model using this protocol. One potential explanation of this is that the model does not fully represent the structural composition of peripheral nerves *in vivo*, in particular the lack of a blood nerve barrier. We hypothesised that MM sera may contain neurotoxic compounds (antibodies, cytokine, other proinflammatory mediators) either different to, or in greater concentration than, POEMS syndrome. Directly exposing peripheral nerves to myeloma sera caused more significant damage over a shorter amount of time. However *in vivo*, such neurotoxic compounds may not be able to access the PNS because of a competent blood nerve barrier. However in POEMS serum, neurotoxic compounds do exist in the serum but at lower concentrations, meaning that damage occurs over a longer timeframe of exposure (and not long enough in our preliminary experiments). We postulated that neuropathy occurs *in vivo* in POEMS syndrome, because the high concentration of VEGF causes disruption to the BNB, facilitating serum access to the endoneuurium and resultant damage. To explore this potential mechanism in more detail, we sought to experimentally prove the previously speculated phenomenon of VEGF causing blood nerve barrier disruption by using the animal blood nerve barrier model described in 6.1.1.9.

6.3 Modelling blood nerve barrier disruption in POEMS syndrome

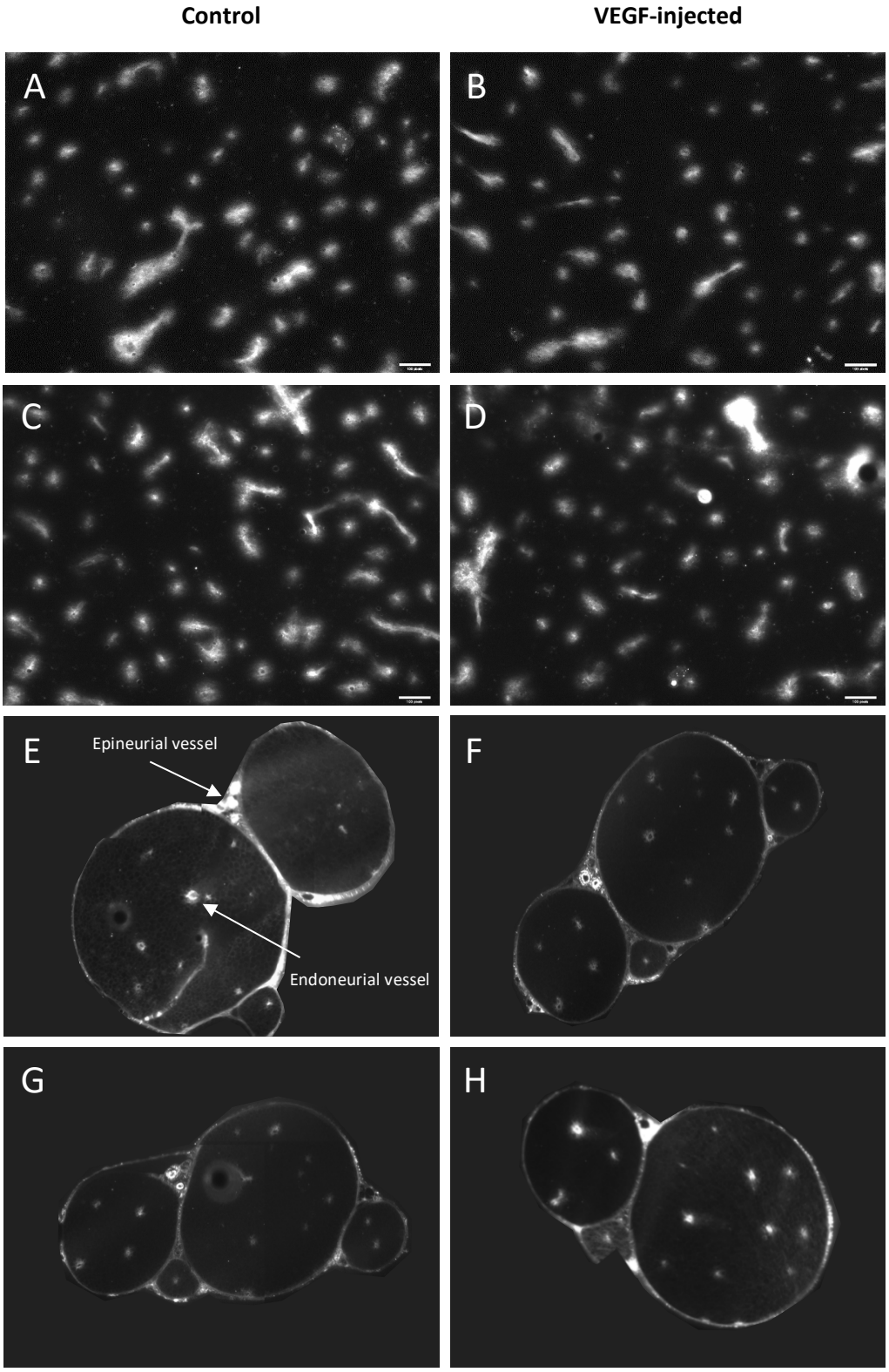
6.3.1 Methods

Four C57BL/6 mice were injected in the lateral tail vein with VEGF 165 cytokine (WHO reference reagent) at 0.9ug per 20g weight. The concentration of VEGF selected was based upon previously reported studies.[384–387] A further 4 mice were injected with the same volume of PBS only. After four hours, 200ul per 20g mouse of Evans blue 1% (Sigma) and 5% BSA (Sigma) were injected into the lateral tail vein, and mice were then sacrificed 30 minutes following. Brain and sciatic nerve were immediately dissected, washed X3 in PBS, snap frozen in liquid nitrogen and stored at -80 ^{o}C. The following day, samples were cut into 10 μm sections using a cryostat and placed onto glass coverslips with fluoromount and imaged immediately using an Axio imager microscope (Zeiss) using the red channel. If imaging demonstrated leakage of Evans Blue through the BnB into the endoneurium, further electron microscopy imaging was performed to provide enhanced detail of tissue structures.

6.3.2 Results

Figure 6.5 demonstrates both cortical (A-D) and sciatic nerve (E-H) blood vessel barrier function following VEGF injection. Evans Blue remained within the blood vessel wall and did not leak into the extravascular space both in the control (PBS) and experimental (VEGF injected) mice in both the cortex (testing blood-brain barrier permeability) and the sciatic nerve (testing blood-nerve barrier). Further optimisation of this experiment will be carried out using different VEGF preparations and isoforms, varying the injected VEGF concentration and the time from injection to harvest. A positive control would also be beneficial to ensure the experiment fundamentally works, and acts as a comparator.

Figure 6.5: Testing blood-brain and blood-nerve barrier permeability of VEGF-injected mice



Fluorescent imaging showing a transverse section of brain cortex (A-D) and sciatic nerve (E-H) from a mouse injected IV VEGF (B, D, F, H) compared to that of PBS control (A, C, E, G) followed by the tracer Evans Blue. Epineurial and endoneurial vessels labelled in E.

6.4 Discussion

This chapter presents two novel experimental techniques to model the peripheral nerve and blood nerve barrier structure and function in both health and disease states. Such methodologies clearly require further optimisation and experimentation before potential neuropathological mechanisms in POEMS syndrome can be identified and replicated.

Exposure of HiPSC myelinated neuronal co-cultures to POEMS syndrome sera did not demonstrate damage beyond that of background degeneration related to environmental factors. The first experiment relied on multiple rounds of imaging to potentially image damage occurring over time compared to a pre-serum-exposed baseline. Although the cell culture system has been shown to remain stable at 90 days, [359] this is when the culture remains in a strictly temperature controlled incubator with minimal interruption, whilst also minimising contamination, maintaining correct humidity and providing stable levels of gases. The process of imaging the 96 wells of a culture plate is time consuming and technically difficult, particularly if multiple fluorescent channels are used. This external confounding factor appeared to impact on neuronal integrity. It was therefore decided to minimise this by incubating plates continuously for one week (other than for two short media changes), and image following fixation only. This appeared to reduce the impact of environmental fluctuations on survival, however despite this, we were still unable to demonstrate damage to the neuronal cultures when exposing the system to neuropathy related samples above that of negative controls. This may be related to exposure time, as cultures may need a longer exposure time to develop changes. Since POEMS serum is thought to cause damage by cytokine mediated

toxicity, it is likely such damage occurs in a dose-dependent manner, meaning damage occurs on reaching either a critical threshold in concentration, or through producing unfavourable conditions with regards to the neuronal microenvironment over time. As POEMS neuropathy develops over weeks or months and continues to progress over time, a requirement for a longer dwell time is possible and consistent with this hypothesis. Also, although we assume that re-exposing the neuronal cultures to fresh disease sera (from the same frozen sample) following media changes would maintain a high level of cytokine exposure, the actual concentration of cytokines within the culture media following exposure was not measured. It may be that the actual concentration of cytokine once diluted in media brings the sample under the pathogenic threshold, although this was why a range of serum concentrations were used (1:5, 1:50 and 1:100). Additionally, neuronal culture media may not provide favourable conditions for cytokines, leading to more rapid degradation to that in the sera, and thus serial testing of cytokine concentrations in the neuronal media would be crucial. In antigen-antibody interactions, which this model has been validated for, binding takes place over seconds to minutes, and thus exposure time is of less relevance. Setting up the same experiment as that in 6.2.2.2, but fixing and imaging at different timepoints i.e. 1 week, 2 weeks, one month and three months for example provides an exposure time more in keeping with what is likely to occur in POEMS syndrome. Regular supernatant sampling and cytokine measurement would be required to ensure high concentrations were maintained.

Another potential area of exploration through using this HiPSC neuronal co-culture model is determining the manner in which neuronal pathology occurs in POEMS syndrome. Neurophysiological studies suggest POEMS neuropathy first develops as a

primarily demyelinating disease with axonal damage occurring as a secondary phenomenon. Using the HiPSC system, it is possible to image neuronal cultures at baseline using fluoromyelin stain, and re-image once permeabilised and fixed using a fluorescent anti-MBP antibody. Overlaying the images allows for visualisation of neurones that have become demyelinated, which can be quantified as a proportion or fold change. Higher power images can also be performed to then study axonal morphology to determine at which stage the axons are involved. An alternative methodology would be to collect the supernatant of such samples and test them for peripherin and periaxin concentrations as markers of axonal and Schwann cell damage (feasible as long as concentrations reach the lower limit of detection for the assay).

One finding which was consistent across both experiments was the significant damage occurring in multiple myeloma samples. This was a surprising finding as myeloma does not directly cause neuropathy. Determining the major ways in which the *in vivo* and *in vitro* systems differ may help to explain this finding. Some of the potential reasons for this were considered, one hypothesis being that the *in vitro* model lacked a functioning blood nerve barrier which allows direct access to normally inaccessible neuronal and glial cells. The presence of an intact blood nerve barrier prevents *in vivo* access to the endoneurium of many mediators of damage. In POEMS syndrome, although the serum is not neurotoxic in this short exposure, toxic mediators may be given access to the endoneurium by factors which open the BNB. VEGF has been proposed by us and others as the most likely candidate to disrupt the BNB in POEMS syndrome, but may also be influenced by other factors such as ICAM-1 which was demonstrated at high levels in POEMS and CIDP compared to myeloma in chapter 5.

To study this phenomenon in more depth, the blood nerve barrier model was utilised to attempt to demonstrate this. Our pilot experiments of injecting VEGF-A isoform 165 ($VEGF_{165,}$) at high concentration into animals did not appear to disrupt the BNB or result in endoneurial or epineurial vessel leak. As before, this experiment was a first attempt and could not be developed further, but the next step would be to consider testing different commercial products of $VEGF_{165,}$ different isoforms, different concentrations and different exposure times. If this were to fail, other putative BNB damaging compounds identified in the sera of POEMS patients such as ICAM1 could be trialled. Demonstrating that such molecules increase permeability of the BNB and/or result in transendothelial movement of serum constituents into the endoneurial space would be key findings supporting both POEMS neuropathy pathology and would support the hypothesis proposed regarding myeloma vs POEMS damage of the HiPSC neuronal co-culture model.

Histopathological specimens of peripheral nerves from POEMS syndrome patients demonstrate epineurial neovascularisation, luminal narrowing and microthrombi clearly demonstrating a significant microvascular angiopathy resulting in vessel leak and likely secondary neurotoxicity. [388] Although epi/endoneurial vessel permeability is demonstrated, the exact mechanism of neuropathy has not been determined. It is conceivable that vessel permeability leading to oedema and inefficient clearance of metabolic by-products is sufficient to produce an unfavourable neuronal microenvironment leading to cell death. This hypothesis in itself could be tested by injecting $VEGF_{165}$ as described, and not only studying the effect this has on vessel permeability, but also to visualise whether this process leads to neuropathology on

electron microscopy. If it does not, VEGF could be injected in combination with other compounds identified in the sera of POEMS syndrome patients to determine whether typical POEMS neuropathology occurs.

Since myeloma samples caused damage to peripheral nerves in the *in vitro* model, it would be useful to explore the composition of myeloma serum in more depth to identify the causative neurotoxic compounds and determine whether they exist in POEMS syndrome. Since the HiPSC model is so new, there are no studies to date which illustrate cytokine mediated neuronal toxicity. Myeloma serum is not solely made up of cytokines, and we should try to demonstrate whether neuronal damage occurs as a result of immunoglobulins, cytokines, or other factors. Myeloma serum can be depleted of immunoglobulins very simply. The normal myeloma and Ig depleted sera could then be tested on the HiPSC neuronal co-cultures to ascertain which fraction causes neuronal damage. Further depletions or additions could be made to isolate and identify pathogenic molecules.

If an experimental methodology could be optimised to demonstrate neuronal damage in the HiPSC model from serum exposure, cytokines could be tested individually for their potential neuropathologic role. Neuronal cultures could be exposed to control serum alone vs control serum spiked with individual cytokines or peptides with increasing concentration, to ascertain whether damage could be identified. This structured and systematic approach may identify key neuropathological markers of POEMS syndrome. In addition, blocking such markers through incubating POEMS syndrome serum with

anti-antibodies then exposing to the neuronal cultures would confirm pathogenicity though lack of causing damage. Such compounds could be potential therapeutic targets for future research.

The two experimental methodologies described in this chapter provide a unique opportunity to study peripheral nerve pathogenesis in high fidelity models, in which experimental variables can be controlled or manipulated to identify key markers of disease. These models provide the opportunity to illustrate the link between the proinflammatory, immune mediated serological markers of POEMS syndrome identified in the previous chapters with the pathognomonic clinical feature of neuropathy. Clearly more work is required to optimise these techniques to produce models which reflect peripheral nerve pathogenesis in POEMS syndrome. This work will likely not only produce discoveries related to POEMS syndrome pathogenesis, but may apply to other forms of inflammatory or immune mediated peripheral nerve disorders.

7. Discussion

7.1 Thesis synopsis

The overarching purpose of this project was to exploit our unique experience in running a highly specialised multi-speciality clinical service, and build a research programme with several complementary elements that could be built upon over several years. This finale chapter summarises the outcomes of my research to date, identifies limitations of the studies, and proposes new directions for exploration.

7.1.1 The natural history of POEMS syndrome

Rich clinical information dating back from 1998 to present day was collected and collated in an accessible customised database, using deep phenotyping to enable characterisation of the disease features, investigations and clinical outcomes. We enrolled 100 POEMS syndrome patients into the study, with patients consenting for prior and future biological samples to be used for research purposes. Collating these data has already enabled clinicians from different specialities to answer important POEMS related questions, and a greater understanding of the disease. Key examples of this are below;

- Identification of a new disease feature of pachymeningitis in POEMS syndrome. [1]
- Enhancing diagnostics through analysing the sensitivity and specificity of VEGF testing in POEMS syndrome. [2]
- Demonstrating the importance (clinically and financially) of early VEGF testing, identifying POEMS cases and instigating treatment. [5]

- Greater detailed knowledge of the presenting features of POEMS neuropathy, distinguishing it from other potential causes, how it progresses over time and recovers following therapy. [3,46]
- Improved understanding of other specific disease characteristics, such as endocrinopathy and clotting abnormalities. [94,95]
- Optimising therapies such as ASCT or anticoagulation. [4,94]
- Identifying risk factors for poor prognosis, enabling clinicians to stratify patients and personalise monitoring and management. [3]

The clinical characterisation, phenotyping and longitudinal sample collection has and will be fundamental in conducting further clinical and laboratory-based research. This is a unique resource for the POEMS research community, allowing the interrogation of blood biomarkers alongside clinical data.

7.1.2 POEMS pathogenesis is typified by hyperinflammation, cytokine and chemokine storms

Utilising the well characterised POEMS syndrome cohort , we were able to test numerous serological samples across different diseases, including POEMS and controls, for 30 cytokines potentially implicated in POEMS pathogenesis. We have discovered that POEMS syndrome sera contains a range of proinflammatory cytokines and chemokines likely to be relevant to pathogenesis or the result of secondary activations 'downstream' of the inflammatory cascade. IL-6, IL-16, IL-7 and VEGF appear to be of most relevance, markedly upregulated in disease, suppressing to normal levels

following treatment and spiking in relapse. The known physiological functions of such cytokines and their proposed pathological relevance in POEMS syndrome is discussed in detail in chapter 4 and summarised schematically in figure 4.5. These findings potentially also explain the lack of clinical efficacy of selective VEGF blockade in POEMS treatment. Several of the cytokines identified in this study had not been implicated before in POEMS syndrome, and their identification adds to the knowledge of the likely underlying disease mechanisms. The combined cytokine panel was also more accurate than VEGF alone at identifying POEMS syndrome cases from other diseases, and thus may be useful clinically when faced with diagnostically challenging situations. Although levels of cytokines and chemokines are significantly raised in active disease, absolute concentrations do not indicate the degree of haematological disease, extent of neuropathy or predict outcome.

7.1.3 Novel protein biomarkers and their potential relevance to neuropathy in POEMS syndrome

Mass spectrometry has allowed for a broadly unbiased exploration of the proteome expressed in the serum of POEMS syndrome patients. Clearly, not all the proteins identified utilising this methodology would be related to the disease itself, however the purpose of this study was to try to uncover novel biomarkers of disease which may be implicated in pathogenesis, or secondary to immune activation or tissue injury. Bioinformatic software packages were used to extract biological meaning from the protein abundance data. POEMS sera contained upregulated proteins predicted to be involved in the Liver X Receptor/ Retinoid X receptor pathway (potentially to downregulate cytokine mediated hyperinflammation), the clotting cascade, the immune

response and the complement cascade. Such pathways were associated with the humoral immune response, inflammatory diseases, cardiovascular and haematological disease, in-keeping with recognised POEMS pathology.

A number of individual biomarkers were identified which appear to be of significance in POEMS pathology. Neuropilin 1 was markedly downregulated in POEMS syndrome; neuropilin 1 is expressed on endothelial cells and acts as a co-receptor for VEGFR1 and VEGFR2, specifically enabling activity of the pathogenic $VEGF_{165}$ isoform. Peripheral nerve-related biomarkers were also identified and are of interest for further study. ICAM1, which was significantly upregulated in both POEMS syndrome and CIDP, has been demonstrated bind to leukocyte ligands in the presence of proinflammatory cytokines and increase the permeability of the blood brain barrier allowing activated leukocytes into the CNS.[389] This protein may thus be involved in the development of pachymeningitis in POEMS syndrome. But of greater possible (but again speculative) relevance is whether ICAM1 also causes similar disruption at the level of the blood nerve barrier, resulting in endoneurial permeability and neuropathy. Neurofilament medium and Tubulin A4 appear to be markers of peripheral nerve damage in POEMS syndrome, and Cofilin and ApoE proteins are likely released following neuronal damage to encourage repair. Research is currently underway exploring the therapeutic potential of elevating cofilin activity in sciatic nerve injury models. [306]

7.1.4 Peripherin, and other axonal biomarkers of peripheral nerve injury in POEMS syndrome

This thesis demonstrated that peripherin is a protein exclusively within peripheral nervous system axons and not in the brain. We have developed a highly sensitive biomarker assay on the SiMOA for peripherin, which demonstrates a broad dynamic range, parallelism, spike recovery and dilutional linearity within acceptable assay limitations. Peripherin has been measured in a range of peripheral nerve conditions, and is found at higher serum levels in more severe axonal diseases. However, levels do not correlate with degree of peripheral nerve severity in POEMS syndrome, and do not reduce following therapy, when other indices for improvement are demonstrated. For these reasons, further optimisation of the assay is required, but this biomarker may be of considerable clinical utility, as described in chapter 3. In addition, this experience has been invaluable in our laboratory, which over the past three years have acquired a SiMOA machine and now have the relatively unique experience of being able to set up and develop homebrew assays for novel biomarkers. In doing so the laboratory plan to expand our peripheral biomarker discovery research in both axonal and demyelinating peripheral nerve proteins.

Neurofilament light (NfL), a well-established but non-specific biomarker of central and peripheral axonal loss demonstrated more expected results. Firstly, levels were increased in POEMS syndrome compared to controls, at lower levels than that of more severe forms of axonal neuropathies. NfL also appeared to reflect disease activity in POEMS syndrome, with levels reducing appropriately following treatment, and correlating with neuropathy measurement scales. In addition, levels of NfL on

presentation with POEMS syndrome correlate with ONLS score at three years following treatment, indicating that patients with greater degrees of axonal damage pre-treatment are more likely to sustain significant longstanding disability. The results of this study suggest NfL is a useful adjunct to the measurement of peripheral nerve injury in POEMS syndrome.

7.1.5 Modelling peripheral nerve damage in POEMS syndrome using a human induced stem cell neuronal co-culture model

The purpose of this final stage of the research was to try to demonstrate a link between the serological changes that take in POEMS syndrome and the pathological processes that take place in the peripheral nervous system. The foreseen benefits of using a HiPSC derived sensory neuron myelinating co-culture system have been discussed in detail in chapter 6. Unfortunately this work was curtailed due to the COVID-19 pandemic. The preliminary data should serve as a platform for future development. We have not been able to demonstrate peripheral nerve damage using the POEMS sera yet, which we suspect may be related to incubation time, or variability in cytokine concentrations. We have however seen frank peripheral nerve damage with myeloma serum exposure, which although unexpected, has possibly illustrated one of the fundamental limitations of such a model given the absence of a blood nerve barrier. This finding has incentivised further work, prompting us to study the blood nerve barrier in more depth with a second model, and investigate serological markers in POEMS serum that disrupt the permeability of the blood nerve barrier leading to neuropathy.

7.2 Limitations

The retrospective longitudinal data collection for the POEMS registry has several limitations. The quality of data retrieval was heavily reliant on the level and quality of detail collected at the time of assessment. This was more common for patients initially assessed by non-neurologists, where neurological examination and assessments were less likely to be performed. A number of patients also received initial investigations at their local hospital prior to being transferred for follow up at UCLH which were not accessible on our systems. In most cases, when patients presented at UCLH for the first time, a comprehensive multi-system assessment with a summary of investigations was documented in computer accessible letters. Information regarding patients' cause of death was also not often recorded, especially as this was often at other hospitals or in the community. In such cases, information was retrieved from GP's records where possible, but detailed information was not always recorded. This information was important for determination of outcome and risk factor analyses. From 2019, all retrospective data collection had been performed, and since then prospective collection is ongoing. In doing so, the multi-specialist clinical team, nursing team and researchers have collaborated to design a proforma to ensure data collection is standardised and encompasses all aspects of clinical and research-based information requirements.

The development of a homebrew biomarker assay, particularly using SiMOA is highly challenging, expensive and time consuming. It is made more difficult when no other methods exist to corroborate levels of the biomarker in clinical samples. At the time of this thesis being written, several limitations remain before the assay can be confidently used to accurately measure peripheral nerve disease.

- The SiMOA machine is temperamental and requires expert care and management. Malfunctions disrupt the development and result in loss of valuable consumables.
- When developing the homebrew assay, the process of conjugating capture antibodies to the paramagnetic carboxylated beads appears to be a key methodological step, and if unsuccessful will negatively influence all subsequent assays. Accuracy and consistency is difficult to achieve with multiple error-prone steps that might adversely affect interassay variances.
- The antibody pair selected for the assay had excellent characteristics in testing but when clinical samples were ran, the results were not entirely as expected from the clinical cases and compared to NfL. As detailed in chapter 3, diluent, matrix effects, or non-specific antibody binding could influence results.

From the data, it is clear that NfL functions as a highly sensitive biomarker of peripheral nerve injury and reflects disease activity. NfL can be used to predict neuropathy outcomes. Arguably, in such circumstances there is no requirement for a second axonal biomarker regardless of it being specific to peripheral nerve damage. It is rare for peripheral and central nervous system diseases to coexist, and thus on most occasions, NfL could be used with a degree of confidence that it is peripherally derived. In addition, possibly due to the antibody combination, but after several years of optimisation and investment, NfL is known to be one of the most sensitive, highly reliable immunoassays Quanterix produces for the SiMOA machine, and thus rivalling this assay with a homebrew is/ will be a considerable feat. However, there are circumstances whereby being able to measure both combined CNS and PNS axonal damage (with NfL) in

combination with a peripheral nerve specific biomarker will be of substantial clinical utility, and hence several research groups are very interested in the outcome of this work. Lastly, the experience gained through the development and optimisation of this immunoassay will be invaluable for future novel biomarker assay designs.

The cytokine discovery and mass spectrometric analysis covered in chapters 4 and 5 have identified a number of molecules we hypothesise to be important drivers of disease in POEMS syndrome. Although outside the scope of this thesis, we have no scientific evidence to prove that the molecules identified play any pathological role in causing direct tissue damage, with particular respect to the characteristic neuropathy. It may be that markers discovered in the blood are secondary to the systemic immune system activation, by VEGF alone, or other unrelated metabolic and systemic processes, whilst nerve specific disease processes remain uncovered. In depth immunohistochemistry of POEMS nerve samples may enable the identification of disease-causing immune cells or specific cytokines within the nerve which would be of relevance.

Another clear limitation of the mass spectrometry work was our failure to use the untargeted shotgun proteomic experiment to create a customised POEMS syndrome multi-plex targeted assay. This would have validated the preliminary mass spectrometry data and possibly also the prior cytokine work (such cytokines could have also been added into the panel) using a high number of patient samples. The pre-configured COVID-19 inflammasome panel measures several proteins not relevant to

POEMS syndrome, and does not include others possibly more useful. However, setting up such a panel requires considerable time, cost and experience, and would require a dedicated thesis in itself.

The HiPSC derived sensory neurones in a myelinating co-culture system is an exciting methodological prospect in peripheral neuroscience, enabling real time imaging of peripheral nerve pathology following exposure to a variety of insults. We hypothesised that this model would enable us to bridge the link between the serological discoveries in chapters 4 and 5 and the peripheral nerve. Clearly more work is required in this area, but a number of observations were made even through preliminary experimentation. Firstly, the procedural steps required to reprogramme and grow out the HiPSC's to neurones requires highly specialised facilities, expertise and time. Although the neuronal culture system has been demonstrated to be stable up to (and likely beyond) 90 days, it became apparent that the system is far less robust when exposed to serum, particularly when taking cultures out of incubation to perform media changes and imaging. The background neuronal damage to cultures may hamper the identification of more subtle neuropathological changes which are taking place. Alternatively, it may be that cytokines (and other disease causing molecules) remain at a stable high concentration in the in vivo peripheral nervous system in POEMS syndrome when there is a continuous supply from the microvasculature; in comparison we do not know what happens to such molecules within sera when then left in a neuronal culture within an incubator. In addition, our data (demonstrating myeloma serum appearing to have a profound effect on peripheral nerve damage, when myeloma does not cause neuropathy *in vivo*) suggested that the absence of a blood nerve barrier may have explained the

discrepancy in results when applying an in vitro model to what may actually occur in vivo. Clearly further work is required to explore and prove such assumptions and is currently underway.

7.3 Future directions

This research project has produced one of the largest, extensively detailed, deeply phenotyped longitudinal cohort databases in POEMS syndrome. These data have already provided invaluable to our understanding of disease, but will also provide a springboard for further clinical and laboratory based research. Our expanding POEMS team of clinicians and researchers meet on a regular basis to discuss our future directions both in research and improving our clinical service, which are detailed below.

As detailed in chapter 3, we currently measure neuropathy in POEMS syndrome using unvalidated clinical disability scales specific to other immune mediated peripheral neuropathies which pose several limitations. We are therefore looking to create a Rasch-built neuropathy focussed clinical outcome measure specific to POEMS syndrome. This will be achieved by formulating a preliminary RODS questionnaire, focused at the activity and participation level, likely utilising sources of information such as that of the International Classification of Functioning, Disability and Health, [113] from semi structured interview with patients and from expert opinion. Items on the preliminary questionnaire will be graded in terms of response, then sending out to a randomly selected subpopulation from the POEMS cohort. Responses will be analysed using Rasch unidimensional measurement methods based upon a mathematical model

proposed by Rasch. [390] The final scale should be unidimensional, where each item is weighted equally with a true sense of precision, a change in the score is measurable and comparable within and between patients. Once developed, the questionnaire can be validated using a separate cohort of the remaining cases, or possibly through collaboration with one of the larger international cohorts. One foreseen potential limitation to this scale is the impact systemic features may have on level of functioning, which may influence the interpretation of the neuropathy score.

A significant number of cytokines have been identified to likely play a role in the pathology of POEMS syndrome and the resulting neuropathy. Thirty cytokines in total were tested, the primary limitation of testing more cytokines being down to cost. Now that the initial data have proved useful, this justifies further exploration of the entire immunological network that appears to play an important role in POEMS pathology. Technologies such as Meso-scale, Luminex and Olink all provide high-throughput multiplex immunoassays of a broad number of biomarkers, some testing to the several thousands. This broad approach may once again identify previously unknown biomarkers of disease activity, or key disease drivers that underpin pathology. More focussed analysis of individual pathways may also be revealing. For example, several cytokines identified were shown to be involved in B cell development and maturation. IL-7 in particular has been shown to stimulate BAFF production. One area of potential interest for future directed study would therefore be the B-cell activating factor/ a proliferation-inducing ligand (BAFF/APRIL) pathway in further detail, expanding upon our preliminary findings.

Determining the origin of cytokine production in POEMS syndrome will be fundamental to our further understanding. Data from prior studies have led us to hypothesise that cytokines are released by both the mono and polyclonal plasma cells within the bone marrow, [109] linking the haematological dyscrasia to the immune system activation, both key factors of POEMS pathogenesis. We are currently applying for ethical committee approval to use prospective, clinically collected bone marrow samples for further research purposes. If permitted, we would look to culture bone marrow cells and measure the supernatant for a range of cytokines. Potentially, sub-populations of bone marrow cells could be separated and independently tested to determine whether differences exist.

The study of the bone marrow of POEMS syndrome patients appears critical to understanding the disease. Several research groups over the previous five years have isolated the monoclonal plasma cell population to typify the genetic and transcriptional landscape of POEMS syndrome. [73,329,391] A number of our hypotheses regarding the production of cytokines, and the role they have on the bone marrow microenvironment and plasma cells have come directly from this thesis and require further exploration. Studies have also demonstrated plasma cell characteristics also play a role in treatment, such as the discovery of cereblon expression being a prognostic marker in POEMS syndrome cases treated with lenalidomide. [392] Although seemingly less relevant to the neurological features of POEMS syndrome, uncovering the origins of disease, factors that propagate the aberrant monoclone, plasma cell dysfunction and cytokine production are likely to assist our understanding of downstream organ dysfunction.

Further refinement of the mass spectrometry methodology is required before strong conclusions can be derived from the data. Further shotgun proteomic study comparing pre- and post-treatment POEMS cases will firstly validate several of the biomarkers already identified in disease, whilst also highlighting whether such compounds suppress following treatment. Proteins that are raised but do not suppress upon treatment are likely to represent separate, unrelated biological pathways. Development of a POEMS syndrome targeted multi-plex proteomic panel, encompassing the proteins identified in this (shotgun and targeted) study, cytokines from chapter 4 and that of the literature is a vital next step. Once more, testing this panel against pre-treated, active, POEMS sera, post-treated stable disease and relapsed sera will clarify the relevant pathological pathways. Further shotgun proteomic exploration of particular disease characteristics, such as testing the sera of patients with and without bone involvement will also potentially identify organ specific markers which can also then be applied to the targeted panel over time.

A number of interesting initial observations were made from utilising the HiPSC derived sensory neuronal culture. Primarily, further investigation into the cause of neuronal damage from myeloma serum would be warranted. Separating antibodies from myeloma serum and repeat testing would potentially determine whether damage is antibody mediated or related to other molecules, likely cytokines or other pathological proteins. If non-antibody mediated damage were the case, exploring the differences within the sera of the myeloma vs POEMS serum would be key to determine the factors which lead to neuronal damage. Identification of the immune cell constituents within the sera of both conditions may also potentially identify neuropathological markers.

Flow cytometry cell separation techniques could be performed to test such cell populations individually on neuronal cultures.

The structure, function and damage of the blood nerve barrier is fundamental pathology in immune-mediated, acquired peripheral neuropathies. We now have a mouse model in which the structure and integrity of the barrier can be explored. Further optimisation of this methodology is required, but it appears that we should be able to test the influence of various cytokines upon the BnB (and the peripheral nerve) through direct injection of recombinant proteins into the mouse systemic circulation. In combination, collaborating with the UCL Medical Research Council Laboratory for Molecular Cell Biology department will enable highly specialised imaging and molecular analyses of POEMS peripheral nerves in disease and regeneration, compared to other immune mediated neuropathies and normal tissue.

Probably of most relevance to patients is the development of new therapeutics. Most POEMS directed therapies are introduced following successful trials in myeloma patient cohorts. Ixazomib therapy is currently being trialled in POEMS syndrome with initially favourable results in pilot studies. [393] Antibody-drug conjugates and chimeric antigen receptor (CAR) T-cell therapies are being directed towards the B-cell maturation antigen for the treatment of plasma cell disorders. [235] BCL-2 the antiapoptotic protein has been demonstrated to be overexpressed in myeloma cells resulting in cell survival, thus Venetoclax, a selective inhibitor of BCL-2 is being trialled in myeloma following success in a number of other haematological malignancies. [394] The myeloid cell

leukaemia-1 (MCL-1) protein is another antiapoptotic member of the BCL-2 family, and subsequent MCL-1 inhibitors are being developed. Interestingly, IL-6, VEGF, BAFF and APRIL have been demonstrated to promote MCL-1 protein expression, which appears consistent with our understanding of POEMS syndrome pathogenesis.[395] Knowledge and reputation of the UCLH POEMS service is expanding, and we are receiving an increasing number of referrals each year of both treatment naive and relapsed POEMS patients for consideration of treatment. We have a responsibility to improve the care that we deliver, and therapeutic trials is certainly an area of development, since we are one of a handful of centres globally with the patient cohorts to be able to conduct such work. This is likely to be more achievable through international collaboration where multiple centres enrol cases for study. Utilising our expertise in neurology, we would also hope to be able to assist trial design through implementing means of measuring disease, using biomarkers such as peripherin or NfL, or clinical outcome measures such as a Rasch built i-RODS for POEMS syndrome.

Lastly, one of our primary objectives of this thesis was to increase physician and patient recognition and understanding of POEMS syndrome. We endeavour to continue to promote patient and public involvement through our locally arranged patient focus groups, to our wider national POEMS syndrome patient day. Although the advent of COVID-19 has greatly interrupted social interaction, particularly for educational events, it has also provoked a boom in videoconferencing, enabling interaction across the globe. We therefore hope to run our subsequent patient day both face to face with our local POEMS cohort, whilst being able to connect with patients who are otherwise more isolated.

7.4 Final conclusion

This thesis characterises the disease features, prognostic factors, treatments and outcomes of a unique cohort of POEMS syndrome cases. In doing so we have identified new disease characteristics, improved diagnostics, identified complications and optimised therapies. We have established a novel biomarker of peripheral nerve injury which may enable more accurate measurement of peripheral neuropathy both in clinical practice and in trials for a range of peripheral nerve disorders. We have advanced our understanding of the complex immune, inflammatory, haematological and coagulation pathways involved in POEMS syndrome both stimulating development and maintenance of the aberrant plasma cell clone and leading to downstream multi-organ damage. Models to study POEMS syndrome peripheral nerve injury have been developed for further exploration. I feel privileged to have had the opportunity to help assemble and work on the highest quality datasets, to combine clinical data with laboratory-based research to further unfold what is an exquisitely rare, highly disabling but fascinating multiple organ system disease.

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Appendix

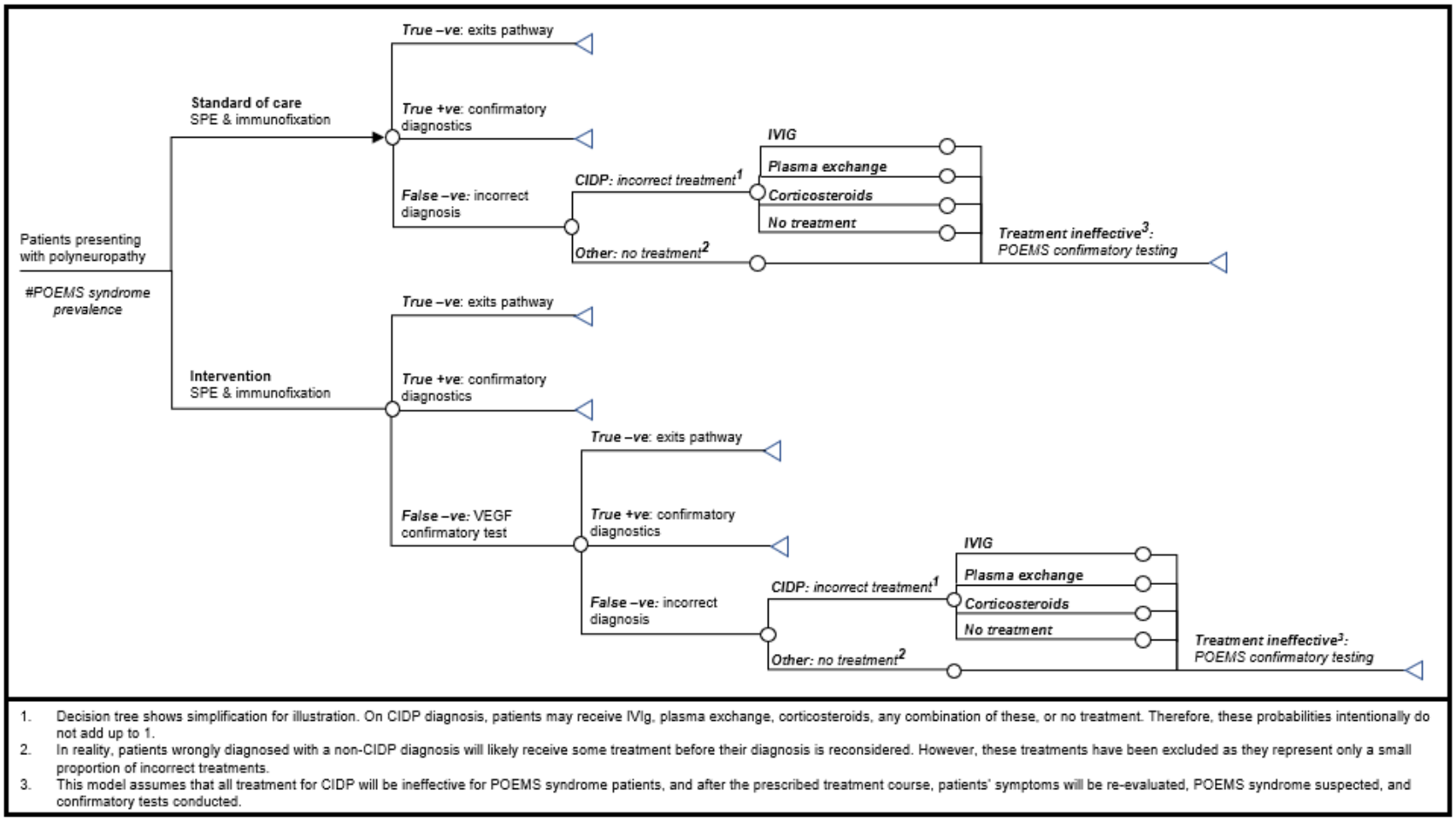
1. POEMS costing analysis

1.1 IVIG costing inputs

	Unit cost (GBP)	Quantity	Cost per course (GBP)
IVIG			
Medication cost	42.50[1]	*Derived*	*Derived*
Delivery costs (personnel and services)	853.00	5	4,265.00
Plasma Exchange			
Drug cost	52.53[2]	21[3]	1,103.12
Delivery cost (personnel and services)	142.00	5	710.00
Corticosteroids			
Drug cost[4]	3.80[5]	4	15.20

1. Cost per kg
2. Octaplas, cost per 200ml
3. 5 treatments of 12ml per kg, 70kg person (4.2 units)
4. Prednisolone (20 mg daily for 4 months); alternatively, patients might be prescribed a course of Dexamethasone (6 months @ 4 x 40 mg tablets). This treatment has been found to have similar outcomes to prednisolone but is much more expensive, and therefore likely used infrequently.
5. Cost per month
Note: All sources included in Supplementary Materials III

1.2 Decision tree model structure



1. Decision tree shows simplification for illustration. On CIDP diagnosis, patients may receive IVIg, plasma exchange, corticosteroids, any combination of these, or no treatment. Therefore, these probabilities intentionally do not add up to 1.
2. In reality, patients wrongly diagnosed with a non-CIDP diagnosis will likely receive some treatment before their diagnosis is reconsidered. However, these treatments have been excluded as they represent only a small proportion of incorrect treatments.
3. This model assumes that all treatment for CIDP will be ineffective for POEMS syndrome patients, and after the prescribed treatment course, patients' symptoms will be re-evaluated, POEMS syndrome suspected, and confirmatory tests conducted.

1.3 IVIG costing inputs and model parameter estimates

Parameter	Mean **base case**	SE	DSA[1] **lower – upper**	PSA[2] **Distribution (α, β)**	Source
Prevalence estimates					
CIDP, as % of patients with inflammatory polyneuropathy	7.2%	0.05*	2 - 10	Beta (4,32)	Calculated (see IV)
POEMS syndrome, as % of patients with inflammatory polyneuropathy	0.8%	0.003*	0.25 - 3.00	Beta (4, 718)	*Calculated (see IV)*
Diagnostic test sensitivity					
SPE	55%	0.05	45 – 65	Beta (54, 45)	Keddie et al.
Immunofixation	78%	0.04	69 – 85	Beta (77, 22)	Keddie et al.
VEGF	94%	0.03	87 – 98	Beta (82, 5)	Keddie et al.
Misdiagnosis rates (on false negative test result)					
CIDP	87%	0.04	76 – 94	Beta (54, 8)	*Estimated from UCLH cohort*
Other	13%	0.04	-	Beta (8, 54)	*Estimated from UCLH cohort*
Treatment for CIDP misdiagnosis (conservative estimate: assuming patients with no treatment info (n=15) received no treatment)					
IVIG	69%	0.06	55 – 80	Beta (37, 17)	*Estimated from UCLH cohort*
Plasma exchange	11%	0.04	-	Beta (6, 48)	*Estimated from UCLH cohort*
Corticosteroids	35%	0.06	-	Beta (19, 35)	*Estimated from UCLH cohort*
No treatment	27%	0.06	-	Beta (15, 39)	*Estimated from UCLH cohort*
Costs					
POEMS diagnosis					
Procedures					
Blood sample	£3.00	-	-	Deterministic	NHS Reference costs [396]
Nerve conduction/ electromyography	£189.00	-	-	Deterministic	NHS Reference costs [396]
Bone marrow biopsy	£177.00	-	-	Deterministic	NHS Reference costs [396]
Bone lesion	£275.84	-	-	Deterministic	NHS Reference costs [396]
PET scan	£470.71	-	-	Deterministic	NHS Reference costs [396]
Tests (test, reagent and personnel costs)					
SPEP	£16.80	-	-	Deterministic	Personal comms [397]
Immunofixation	£44.80	-	-	Deterministic	Personal comms [397]
VEGF	£55.00	-	-	Deterministic	UCLH Neuroimmunology Handbook [398]
Bone marrow biopsy processing	£290.00	-	-	Deterministic	Personal comms [399]
Consultations					
GP appointment	£37.00	-	-	Deterministic	2018 Unit Costs of Health and Social Care [160]
Consultant-led first clinic appointment	£211.00	-	-	Deterministic	NHS Reference costs [396]
Consultant-led follow-up clinic appointment	£221.00	-	-	Deterministic	NHS Reference costs [396]
Treatment for CIDP misdiagnosis					

Drug costs					
IVIG cost per gram	£42.50	-	£14.17 - £127.50	Deterministic	2016 NHS Policy Document [400]
PE (Octaplas, cost per 200ml)	£220.63	-	-	Deterministic	Open Prescribing database [401]
Prednisolone (20 mg, 30 tablets)	£3.80	-	-	Deterministic	Open Prescribing database [401]
Treatment variables					
IVIG days per course	5	2.5*	2 – 7[2]	Gamma (4, 1)	Gorson et al. 2012 [402]
Number of treatments for per course of PE	1	0.5*	2 – 7[2]	Gamma (4, 1)	Gorson et al. 2012 [402]
Number of courses of PE	5	2.5*	1 – 4[2]	Gamma (4, 0)	Gorson et al. 2012 [402]
Hospital charges					
Outpatient admission for IVIG	£853.00	-	-	Deterministic	NHS Reference costs [396]
Outpatient admission for PE	£142.00	-	-	Deterministic	NHS Reference costs [396]

1. Deterministic Sensitivity Analysis
2. Probabilistic Sensitivity Analysis

1.4 Prevalence estimates

We used CIDP and POEMS syndrome prevalence among patients presenting with an inflammatory polyneuropathy, rather than national estimates, to match the starting cohort of the decision-tree. We obtained an estimate of the proportion of polyneuropathy cases presenting to a hospital with an inflammatory polyneuropathy, polyneuropathy and CIDP incidence rates, and CIDP and POEMS prevalence rates from the literature.[100,403] We divided CIDP incidence by our calculated inflammatory polyneuropathy incidence to estimate the proportion of inflammatory polyneuropathy patients with CIDP and used the prevalence rate ratio of CIDP to POEMS to approximate the proportion of inflammatory polyneuropathy patients with POEMS syndrome in our base-case.

	Per 100,000	Incident cases	%
National polyneuropathy incidence	77	40,391[1]	
Inflammatory polyneuropathy incidence (% of polyneuropathy)		3,635[2]	9.0
National CIDP incidence	0.5	262[1]	
National CIDP prevalence	2.8		
National POEMS syndrome prevalence	0.3	28[1]	
CIDP prevalence among patients presenting with an inflammatory polyneuropathy			7.2[3]
POEMS prevalence among patients presenting with an inflammatory polyneuropathy			0.8[4]

1. **Case calculated by multiplying incidence rate multiplied by UK adult population**
2. **Calculation: national polyneuropathy incidence * % polyneuropathy cases that are inflammatory**
3. **Calculation: CIDP incidence (262)/ National inflammatory polyneuropathy incidence (3,635)/ *100**
4. **Calculation: CIDP as % of inflammatory polyneuropathy (7.2%)* prevalence rate ratio POEMS (0.3): CIDP (2.8)**

1.5 Cohort demographics and outcome analysis

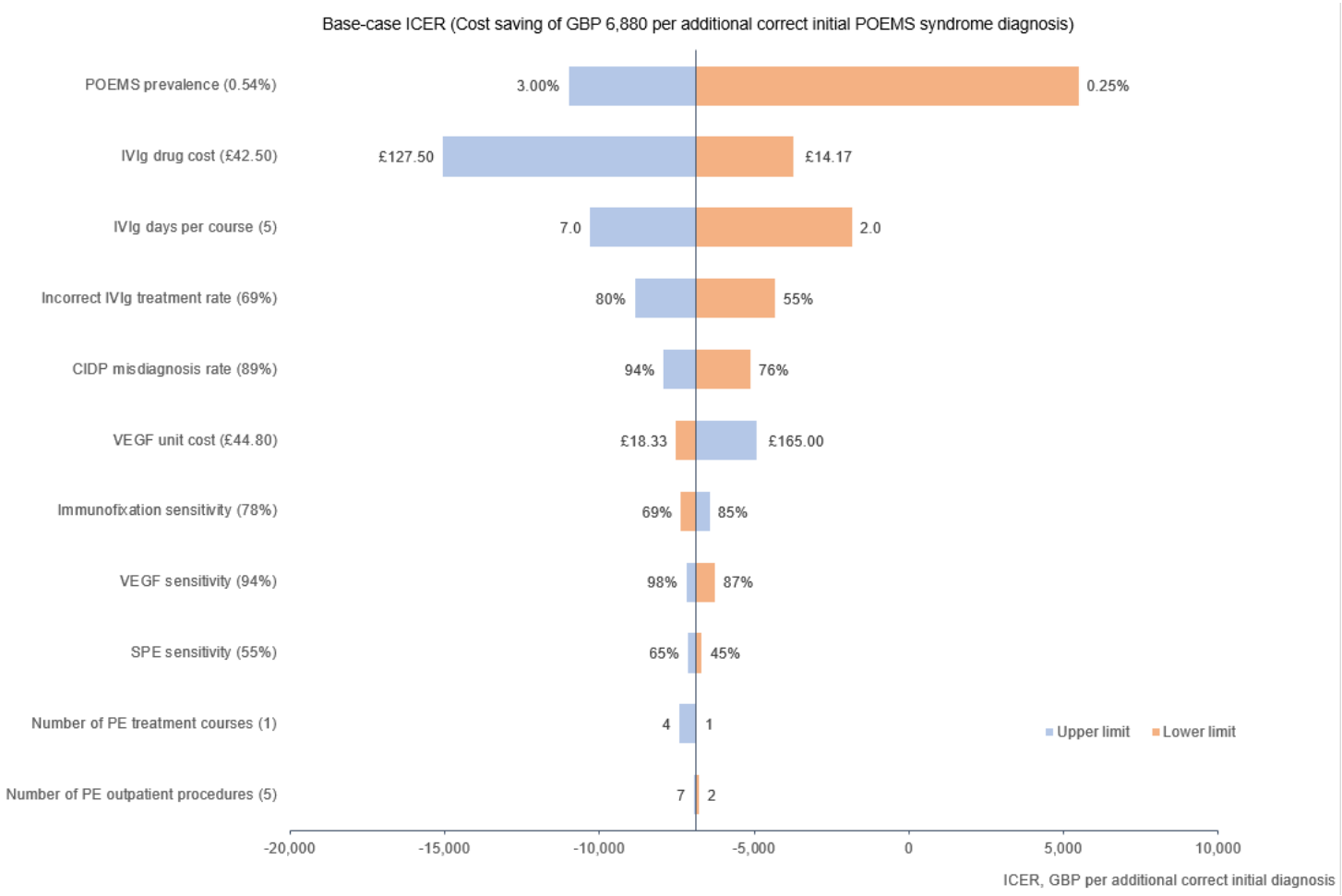
Variable	N	All (95% CI/ IQR)[1]	Direct (95% CI/ IQR)	Indirect - CIDP (95% CI/ IQR)	Indirect -other (95% CI / IQR)	p value
Descriptive statistics						
Cohort size (%)	100	-	37 (28 – 47)	55 (45 – 65)	8.0 (4.0 – 15)	-
Males (%)	69	69 (60 – 78)	65 (48 – 79)	71 (57 – 81)	75 (37 -94)	-
Age at diagnosis (mean)	100	55 (52 – 57)	55 (50 – 61)	53 (50 – 57)	59 (45 – 73)	0.78[2]
Waiting time						
Symptoms to diagnosis, months (median)	100	11 (7.0 – 21)	9.0 (6.0 – 13)	14 (7.0 – 24)	8.0 (6.1 – 13)	0.11[3]
Pre-diagnosis						
Total no of symptoms (mean)	100	7 (3.0 – 12)	7.1 (6.4 – 7.7)	7.1 (6.6 – 7.5)	6.5 (5.0 – 8.0)	0.67[2]
Mobility score (median)	99	3.6 (3.0 – 5.0)	3.0 (1.5 – 5)	4.0 (3.0 – 5.0)	3.5 (3.0 – 5.0)	0.38[3]
Wheelchair/ bedbound (%)	99	37 (28 – 48)	36 (20 – 53)	38 (25 – 51)	18 (0.0 – 81)	0.98[4]
ONLS (median)	100	6 (4.0 – 8.0)	5.0 (3.0 – 8.0)	7.0 (4.0 – 9.0)	6.5 (4.0 – 8.5)	0.13[3]
Post-diagnosis						
Mobility score (median)	99	1.5 (1.0 – 3.5)	1.5 (1.0 – 3.5)	3.0 (2.0 – 4.0)	3.0 (1.5 – 3.0)	0.10[3]
Wheelchair/ bedbound (%)	99	12 (6.4 - 20)	8.3 (0.0 – 17)	16 (6.3 – 26)	0 (0 – 0)	0.26[3]
ONLS[5] (median)	52	4.0 (3.0 – 5.0)	3.5 (1.5 – 5.5)	4.0 (2.0 – 5.0)	4.0 (4.0 – 5.0)	0.84[3]
Clinical response (%)	56	65 (52 – 77)	54 (33 – 76)	69 (52 – 86)	100 (100 – 100)	0.26[4]
Haematological response (%)	89	48 (38 – 59)	53 (35 – 71)	47 (32 – 61)	33 (0 .0 – 88)	0.64[4]
VEGF response (%)	90	79 (69 – 87)	71 (54 – 87)	82 (70 – 93)	100 (100 – 100)	0.24[4]
Relapse (%)	100	32 (23 - 42)	32 (17 – 48)	35 (22 – 48)	13 (0.0 – 42)	0.40[4]
Mortality (%)	100	12 (6.3 – 20)	13 (2.0 – 25)	13 (3.6 – 22)	0 (0.0 – 0.0)	0.91[4]

Abbreviations: CI=Confidence Interval; IQR=Interquartile Range

1. **95% CI shown for means or proportions, IQR shown for median values**
2. **ANOVA test statistic**
3. **Kruskal-Wallis H test statistic**
4. **Likelihood Ratio (LR) Chi-Square test**
5. **ONLS measured 3 years after treatment completion**

1.6 Cost effectiveness analysis

Figure 1: Deterministic sensitivity analyses of Incremental Cost-Effectiveness Ratios (ICERs) for Intervention vs Current Standard of Care.



2. Protocols

2.1 Rat dissection and homogenisation protocol

Materials

1. Dissecting tools:
    a. Forceps - curved blunt forceps with serrated tips
    b. Surgical scissors
    c. Scalpel (size-11 blade)
2. Dissecting board
3. 4% (v/v) isoflurane, 70% ethanol
4. Pins & tissue paper
5. Foil
6. Dry ice
7. Weighing scale
8. Eppendorf tube 1.5ml
9. Mechanical tissue homogeniser
10. Tris buffered saline (TBS)
11. Protease inhibitor- Halt protease inhibitor cocktail, Thermofisher (cat number 87785

Methods: Dissection of sciatic nerve

1. Pin rat's limbs to dissection board, clean with isoflurane and ethanol sprays

2. Lay foil onto dry ice so freezes
3. Using surgical scissors and forceps cut skin at infero-lateral aspect of hindleg up to the lumbar spine. Separate muscles and isolate sciatic nerve. Separate adjoining tissues.
4. Cut sciatic nerve as proximal to spinal column as possible down to distal segment at posterior knee.
5. Wash sciatic nerve x3 with TBS
6. Lay nerve onto frozen foil and will freeze immediately.
7. Repeat for contralateral sciatic nerve

Dissection of other neurological and non-neurological tissue

1. The rat brain was dissected according to predefined protocols. [180]
2. A midline incision was made from the neck to the urethra and the skin pulled back and pinned. The heart, lungs, spleen, kidneys, liver and pancreas were all dissected using anatomical guides. [181]
3. The spinal column was subsequently dissected by cutting laterally along the transverse spinous processes. To enter the spinal canal an incision was made at the lamina. Scissors were used to cut each lamina and the spinal colum then opened using forceps. Scissors were then used to cut 2cm of spinal cord tissue cranially and caudally, then to separate the roots. Once free the spinal cord was removed using forceps.

Tissue homogenisation- protocol adapted from Abcam guide. [182]

1. Weigh tissue then insert into Eppendorf tube. If tissue weight over 2 grams then cut tissue to 2g weight maximum.
2. Add ice told TBS solution at 1ul per ug tissue and protease inhibitor 5ul per 100ug tissue
3. Use mechanical homogeniser maintaining constant agitation for 10 minutes until tissue fully broken down in solution.
4. Leave on ice for 30 minutes
5. Centrifuge at 12,000g for 30 minutes at 4 degrees. Aspirate supernatant and place in fresh Eppendorf tube.
6. Perform bicinchoninic acid (BCA) assay to determine protein concentration.
7. Freeze sample at -80 oC.

2.2 Dot blot protocol

Materials

1. Nitrocellulose membrane
2. Distilled water
3. 5ml weighing boats
4. Antibodies of choice
5. Recombinant protein and/or tissue homogenate
6. Bio rad chemi doc imaging system

Method

1. 2ul of sciatic nerve homogenate, recombinant (both at 1ug/ul) or control (ddH20) was added to nitrocellulose membrane and left for 30 minutes to dry.
2. Membranes were blocked using PBS in 5% BSA for 1 hour.
3. Membranes were then soaked in primary antibody solution at varying concentrations in 5% BSA for 30 minutes.
4. Membranes were washed for 3 x 5 minutes using PBS-tween 0.05%
5. HRP-conjugated anti-species antibody was added at 1:5000 dilution in 5% PBS BSA for 30 minutes.
6. Membranes were washed for 3 x 5 minutes using PBS-tween 0.05%
7. Membranes were developed using electrochemiluminescent (ECL) reagent and imaged immediately with ECL camera.

2.3 Western blot Sample preparation for loading into gels

Materials

1. Denaturing agent LDS (4x)- NuPAGE (cat number 1879570)
2. Dithiothreitol Reducing agent (x10)- NuPAGE (cat number NP0009)
3. Tissue homogenate
4. Distilled water
5. Heat block

Method

1. Aim for 30ul total volume of loading sample per western blot lane
2. Add 7.5ul of LDS, 3ul of DTT and load appropriate volume of tissue sample to provide 30ug protein into one Eppendorf tube.
3. Make up rest of volume to 30ul using distilled water.
4. Place sample in heat block at 65 oC for 10 minutes.

2.4 Western blot procedure – protocol adapted from Abcam guide. [404]

Materials

1. Molecular weight marker- Invitrogen magicmark western standard (cat number LC5602)
2. Western blot gel- Nupage 4-12% Bis-Tris Protein Gel, 1mm, 10 lane (cat number NP0321BOX)
3. X Cell Surelock Mini-Cell Electrophoresis system
4. Transfer buffer - X20 NOVEX NUPAGE (cat number NP0006-1 1878558)
5. Running buffer - X20 NOvEX NUPAGE (cat number NP0001 1927981)
6. Methanol - VWR chemicals (cat number 20846.326)
7. Commassie blue dye - Thermofisher (cat number 20278)
8. Ponceau 1% - Sigma (cat number P7170)
9. PBS BSA 5%
10. PBST 1%

11. PBS
12. Primary and secondary antibodies (see table 8)
13. Rabbit anti mouse antibody - Dako (cat number P0260)
14. Swine anti-rabbit antibody - Dako (cat number P0399)
15. ECL reagent – Thermofisher Supersignal chemiluminescent (cat number 34580)
16. Bio-rad ECL image analyser

Method

1. Place gel in western blot tank and fill with running buffer
2. Add 5ul of magicmark to lane 1 and 30ul of loading samples to SDS-PAGE wells.
3. Run gel at 200v for 45 minutes.
4. Transfer using 450ul distilled water, 25ml transfer buffer and 25 ml methanol.
5. Set up transfer as below:

6. Transfer at 4 oC in cold room at 25v for 2 hours.
7. Block membrane with PBS 5% BSA for 30 minutes
8. If using different antibodies to stain can cut lanes of membrane at this stage and separate membranes into individual weigh boats.
9. Cover membrane in 1/1000 antibody solution in PBS 5% BSA
10. Wash 5x5 minutes with PBS-tween 0.1%
11. Add secondary HRP conjugated antibody at 1/5000 for 2 hours.
12. Wash 5x5 minutes with PBS-tween 0.1%
13. Add ECL read buffer to cover membranes
14. Read using Bio-Rad ECL image analyser or Alpha Innotech FlorChem SP Gel Imaging System

2.5 Peripherin ELISA protocol

Materials

1. Coating buffer- Calcium carbonate buffer Ph 9.91
2. Blocking solution- 5% skim milk in PBST 0.05% Tween 20
3. Diluent- 2% skim milk in PBST 0.05% Tween 20
4. Wash- PBST 0.05% Tween 20
5. Coating antibody- MC6 (see table 5)
6. Recombinant protein 3 0.17 mg/ml stock (see table 5)
7. Detector PC2 (see table 5)
8. Secondary HRP conjugated swine anti-rabbit (table 5)

9. Substrate- TMB solution (thermofisher Cat #34028)
10. Stop solution- H2S04 1M
11. Plate- Uncoated Nunc maxisorp 96 well plate (Cat 44-2404-21)

Methods

1. Apply 100ul capture antibody at 1:3000 to wells in coating buffer, shake for 5 minutes at 600rpm for 5 minutes then incubate overnight in cold room 4 degrees.
2. Discard capture antibody, add 100ul of blocking solution per well, incubate on shaker at 600rpm at room temp for 1 hour
3. Discard blocking solution and wash plate with PBST 0.05% Tween 20 once. Blot plate on tissue paper to remove excess.
4. Dilute recombinant protein to 1/500 highest concentration of 340,000pg/ml and perform 1:5 dilution to provide concentrations of 68000, 13600, 2720, 544, 108 and 21.6pg/ml and blank of PBS only. Add 100ul to wells in duplicate to form standard curve.
5. Vortex for 5 seconds then spin serum sample on benchtop centrifuge prior to use. Apply 100ul per well undiluted. Incubate on shaker at 600rpm at room temp for 1.5 hours.
6. Discard samples and calibrators. Wash 3x with PBST and blot on tissue paper to remove excess.
7. Apply detection antibody at 1:2000 concentration using diluent at 100ul per well. Incubate for one hour on shaker at 600rpm at room temp.
8. Discard detector antibody. Wash 3x with PBST and blot on tissue paper to remove excess.

9. Add 100ul per well of secondary HRP conjugated antibody at 1/5000 to all wells. Incubate at room temp on shaker at 600rpm for 1 hour.
10. Discard secondary HRP antibody. Wash 5x with PBST and blot on tissue paper to remove excess.
11. Add 100ul per well of substrate (TMB), incubate in the dark for 15 minutes
12. Add H2SO4 1M 100ul per well to stop reaction
13. Read absorbance at 450nm on plate reader

2.6 Peripherin MSD protocol

Reagents

1. Coating buffer- PBS
2. Blocking solution-PBS 5%Bovine Serum Albumin (BSA)
3. Antibody diluent- PBS 1% BSA
4. Secondary sulfotag diluent- purified water
5. Wash- PBST 0.05% Tween 20
6. Coating antibody- MC6 (see table 5)
7. Standard- recombinant protein 3 (see table 5)
8. Detector- PC2 (see table 5)
9. Secondary sulfotag goat anti rabbit antibody (Cat R32AB-1)
10. Substrate- MSD read buffer solution (MSD Cat #R92PC-2)
11. Plate- Quickplex 96 well plate (MSDL55XA)

Assay protocol

1. Apply 40ul capture antibody diluted to 2ug/ml in PBS to wells, shake for 5 minutes at 600rpm for 5 minutes then incubate overnight in cold room 4 degrees.
2. Discard capture antibody, add 100ul of blocking solution per well, incubate on shaker at 600rpm at room temp for 1 hour
3. Discard blocking solution and wash plate with PBST 0.05% Tween 20 once. Blot plate on tissue paper to remove excess.
4. Dilute recombinant protein to 1/500 highest concentration of 340,000pg/ml and perform 1:5 dilution to provide concentrations of 68000, 13600, 2720, 544, 108 and 21.6pg/ml and blank in PBS only. Add 25ul of protein to wells to form standard curve.
5. Vortex for 5 seconds then spin serum sample on benchtop centrifuge prior to use. Apply 25ul per well undiluted. Incubate on shaker at 600rpm at room temp for 2 hours.
6. Discard samples and calibrators. Wash 3x with PBST and blot on tissue paper to remove excess.
7. Apply detection antibody at 1:4000 concentration using diluent at 25ul per well. Incubate for one hour on shaker at 600rpm at room temp.
8. Discard detector antibody. Wash 3x with PBST and blot on tissue paper to remove excess.
9. Add 100ul per well of sulfotag conjugated antibody at 1/2000 diluted in purified water to all wells. Incubate at room temp on shaker at 600rpm for 1 hour.
10. Discard secondary antibody. Wash 5x with PBST and blot on tissue paper to remove excess.
11. Add 100ul per well of read buffer using reverse pipette to avoid bubbles
12. Read plate on MSD imager

3. Buffers

3.1 Immunoprecipitation buffer

Stock	Formulation and Vol	Final conc	Volume for 5 ml
1M Tris-HCL (x4) T1503	6.05 of Tris Buff to 7.3 End vol **50m**L	25mM	125 ul
2000mM NaCl (x7.3) S9625	2.92 g 50ml ddH20 End vol **50m**L	137mM	342 ul
10% NP-40 (x14) NP40-s	7.14ml 42.86ml ddH20 End vol **50m**L	0.5%	250ul
50% Glycerol (x50) G5516	25ml and 25ml of ddH20 End vol **50m**L	10%	1000ul
Protease inhibitor (1:100) P8340	Ready to use		50 ul
ddH2O			3233ul

3.2 Tissue lysis buffers

Name protocol	Stock	Formulation	Final conc	Vol for 1ml
Protocol 1 Buffer A	Tris 1M pH 7.5	1.2114g in 10ml H20	Tris 10mM	10 uL in 1ml solution
	NaCL 2000mM	5.84g NaCl make up to 50ml H20	NaCl 500mM	250ul in the total 1ml solution
	10% Triton X100 in 50ml	5ml Triton X in 45ml dist H20	Triton X100 0.1%	10ul of stock 10%
	10% BME		1% BME	100ul if of 10% stock
			PI cocktail 1:100	10ul
			Water	1000-452 (total)=548
Protocol 1 Buffer B	15M urea	18.08g make up in 20ml dist H20	7Molar urea	466ul stock in 1ml
	5M thiourea	9.51g in 25ml	2M Thiourea	400ul stock in 1ml
	2g in 10ml CHAPS		5% CHAPS	200ul in 1ml
				50ul dist H20
Protocol 1 Buffer C	Laemmli buffer-Thermofisher 4x		Laemmli buffer-Thermofisher 1x	30ul well / 4 =7.5ul
	DTT 1:10			30ul/well so use 3ul per well
Protocol 2 Buffer A	Tris 1M pH 6.8	1.2114g in 10ml H20	50mM Tris HCL 6.8pH	50ul of stock in 1ml
	10% Triton X-100	5ml Triton X in 45ml dist H20	1% Triton X	100ul of 10% Triton
	50% glycerol	10ml in 10ml H20	10% glycerol	200ul of the 50% solution
	Got NaCL 2000mM	5.84g NaCl	0.15M NaCL	75l of stock in 1ml

		make up to 50ml H20		
	200mM EDTA	0.584g in 10ml H20	2mM EDTA	10ul of stock in 1ml
			PI inhib 1:100	10ul in 1ml
				+515ul water
Protocol 2 Buffer B	Laemmli buffer- Thermofisher 4x		Laemmli buffer- Thermofisher 1x	30ul well / 4 =7.5ul
	DTT 1:10			30ul/well so use 3ul per well
Protocol 3 Buffer A	Tris 1M pH 7.5 already got	1.2114g in 10ml H20	25mM Tris pH 7.5	25ul in 1ml
	Got NaCL 2000mM	5.84g NaCl make up to 50ml H20	50mM NaCl	25ul in 1ml
	10% Triton X-100	5ml Triton X in 45ml dist H20	1% Triton X	100ul of 10% Triton
	PI cocktail 1:100			PI cocktail 1:100 10ul in 1ml
Protocol 3 Buffer B	Tris 1M pH 7.5	1.2114g in 10ml H20	25mM Tris pH 7.5	25ul in 1ml
	NaCL 2000mM	5.84g NaCl make up to 50ml H20	50mM NaCl	25ul in 1ml
	SDS 10%	2.5g in 25ml	SDS 5%	500ul in 1ml
Protocol 4	1g tissue to 10ml NPER			