THE UTILITY OF INTACT LIGHT CHAIN MASS SPECTROMETRY-BASED ASSAYS FOR THE DETECTION OF PLASMA CELL DISORDERS AND THE ASSESSMENT OF TREATMENT RESPONSES

By

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A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

Institute of Immunology and immunotherapy

The University of Birmingham

June 2023

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Abstract

Mass spectrometry (MS) assays have been emerging over the last few years as a new and potentially more sensitive way to track monoclonal proteins in the serum patients with plasma cell disorders (PCD), although limited data on their prognostic utility is available. In addition, no studies have explored their potential utility in patients with multiple myeloma with low-level monoclonal protein production. Despite the use of intact light chain MS-based assays in lieu of immunofixation being approved by the Internal Myeloma Working Group in 2021, MS testing to monitor patients with PCD is currently only available at a single institution who have developed an in-house test. This thesis presents work undertaken alongside the Binding Site Ltd to develop a regulatory approved intact light chain MS-based assay and clinical studies undertaken independently to assess the utility of this assay for monitoring patients with PCD.

Assay optimisation work conducted as part of this thesis has shown that the combination of free light chain (FLC) specific reagents in addition to a five bead matrix-assisted laser desorption/ionisation time-of-flight MS (MALDI-TOF MS) assay provides a more sensitive panel for the detection of low-level residual monoclonal FLC. However, the application of MALDI-TOF MS to urine did not provide any additional sensitivity to the serum-based MALDI-TOF MS-based assays. The sensitivity of FLC-MS for the detection of low-level residual monoclonal FLC was also enhanced by on-bead de-glycosylation in patients with light chain N-linked glycosylation.

The first clinical study included in this thesis explored the sensitivity and prognostic utility of MALDI-TOF MS in patients being treated with carfilzomib, cyclophosphamide, lenalidomide, and dexamethasone (KCRD) in the Myeloma XI trial. In this work MALDI-TOF MS provided greater sensitivity for the detection of residual monoclonal protein compared to electrophoretic techniques and MALDI-TOF MS negativity was associated improved progression free survival (PFS). In addition, FLC-MS negativity in patients with no residual monoclonal protein

detection by immunofixation electrophoresis at the end of induction, day+100 post autologous stem cell transplant (ASCT) and six months post maintenance randomisation was associated with reduced PFS.

The second clinical study evaluated the utility of MALDI-TOF MS in patients with non-measurable myeloma. This study showed that 91% of patients classified as having non-secretory myeloma using standard techniques have detectable monoclonal protein in the serum using MALDI-TOF MS. MALDI-TOF MS also provided greater sensitivity for monitoring patients with oligo-secretory myeloma. Further studies should therefore be undertaken to establish criteria for measurable disease using MS.

The third clinical study included in this thesis evaluated the utility of a FLC specific MALDI-TOF MS assay in patients undergoing treatment for AL amyloidosis in the ALchemy study. This is first study to evaluate the prognostic utility of a FLC specific MS-based assay in patients with AL amyloidosis and found that achieving FLC-MS negativity at six and 12 months post treatment initiation was associated with improved overall survival. There was also an improved rate of renal responses amongst patients who achieved FLC-MS negativity at 12 months.

The final clinical study included in this thesis is the first study to evaluate the prognostic significance of light chain N-linked glycosylation in patients undergoing treatment for PCD. This study included patients treated with KCRD and ASCT for multiple myeloma and bortezomib-based treatment for AL amyloidosis. In contrast to the higher risk associated with light chain N-linked glycosylation in patients with monoclonal gammopathy of undetermined significance previously described by Dispenzieri *et al* this work found that light chain glycosylation was not associated with inferior responses to treatment or reduced survival.

Acknowledgements

I would like to thank my supervisors Professor Guy Pratt and Dr Simon North for their mentoring and guidance during my PhD and The Binding Site Ltd part of Thermo Fisher Scientific® for providing funding for the mass spectrometry testing. I would also like to thank Miss Nicola Wright and Dr Sabah Pasha for the training they provided on the interpretation of mass spectrometry data.. In addition, I would like to thank the collaborators from the UK Myeloma Trial Management Group, Professor Wechalekar and team at the National Amyloidosis Centre and the Australian Leukaemia and Lymphoma Group and the patients for providing residual serum samples for analysis during this project. Lastly, I would also like to thank my husband Wayne, for his constant support and patience during this 4 year marathon!

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PUBLICATIONS ARISING FROM THIS THESIS

Peer-reviewed Publications

Giles HV, Drayson MT, Kishore B, Pawlyn C, Kaiser M, Cook G *et al*. Progression free survival of myeloma patients who become IFE-negative correlates with the detection of residual monoclonal free light chain (FLC) by mass spectrometry. *Blood Cancer Journal* 2024; 14(1): 50.

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Conference Abstracts – Oral Presentation

Oral presentation at ASH 2022 – abstract presented by J. Bomsztyk

Bomsztyk J, Ravichandran S, **Giles HV**, Berlanga O, Harding S, Khwaja J *et al*. Use of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) free light chain mass spectrometry for the diagnosis and monitoring of systemic immunoglobulin light chain (AL) amyloidosis. *Blood* 2022; 140(S1): 2351-2353.

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Giles HV, Drayson MT, Wright N, Cook G, Davies FE, Morgan GJ *et al*. Residual Monoclonal Free Light Chain Positivity by Mass Spectrometry Identifies Patients at

Increased Risk of Early Relapse Following First-line Anti-myeloma Treatment. *Blood* 2021; 138(S1): 820.

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Giles HV, Wright NJ, Pasha S, North S, Booth, S, Harding *et al***.** On bead deglycosylation coupled with MALDI-TOF mass spectrometry provides a simple method for confirming light chain glycosylation and provides a sensitive method for residual disease detection. https://www.isaamyloidosis.org/assets/docs/ISA22_Abstract%20Book_final.pdf

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ABBREVIATIONS

ACR Albumin: creatinine ratio

AIB Antibody independent binding

AKI Acute kidney injury

AL amyloidosis Light chain amyloidosis

ALLG Australian Leukaemia and Lymphoma Group

ALP Alkaline phosphatase

ASCT Autologous stem cell transplantation

BCMA B-cell maturation antigen

BJP Bence Jones protein

BR Bendamustine rituximab

BSH British Society of Haematology

CDR Cyclophosphamide, dexamethasone and rituximab

CR Complete response

ctDNA Circulating tumour DNA

CTD Cyclophosphamide, thalidomide and dexamethasone

CTDa Attenuated cyclophosphamide, thalidomide and dexamethasone

D Diversity

dFLC Difference between the uninvolved and involved free light chain

DW-MRI Diffusion-weight magnetic resonance imaging

eGFR Estimated glomerular filtration rate

FISH Fluorescence in situ hybridization

FLC Free light chain

HCCA alpha-cyano4--hydroxycinnamic acid

ICC Intraclass correlation coefficient

IFE Immunofixation electrophoresis

iFLC Involved free light chain

IgH Immunoglobulin heavy chain

IMiD Immunomodulatory drug

IMWG International Myeloma Working Group

ISA International Society of Amyloidosis

ISS International Staging System

IVD In vitro diagnostic test

IZKF Ikaros family zinc finger

J Joining

KCRD Carfilzomib, cyclophosphamide, lenalidomide and Dexamethasone

KTD Carfilzomib, thalidomide and dexamethasone

LC-MS Liquid chromatography-mass spectrometry

LDH Lactate dehydrogenase

LOD Limit of detection

MALDI-TOF MS Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

MGUS monoclonal gammopathy of undetermined significance

MRD Minimal residual disease

m/z Mass-to-charge ratio

MS Mass spectrometry

MTA Material transfer agreement

nCR Complete response without bone marrow confirmation

NFKB Nuclear factor kappa-B

NGF Next generation flow cytometry

NGS Next generation sequencing

NSMM Non-secretory multiple myeloma

NT-proBNP N-terminal prohormone of brain natriuretic peptide

OS Overall survival

OSMM Oligo-secretory multiple myeloma

PBST Phosphate buffered saline with 0.1% TWEEN 20

PCD Plasma cell disorders

PET-CT Positron emission tomography computed tomography scan

PF4 Platelet factor 4

PFS Progression free survival

PNGase F peptide :N glycosidase F

PR Partial response

QIP-MS Quantitative immune precipitation mass spectrometry

RAG Recombination activating gene

RCD Lenalidomide, cyclophosphamide and dexamethasone

R-ISS Revised International Staging System

sCR Stringent complete response

SDW Standard deionised water

SPE Serum protein electrophoresis

TCEP Tris(2-carboxyethyl)phosphine)

TEAB triethylammonium bicarbonate

uFLC Uninvolved free light chain

UHB University Hospitals Birmingham NHS Foundation Trust

V Variable

VCD Bortezomib, cyclophosphamide and dexamethasone

VGPR Very good partial response

V/V volume for volume

1:INTRODUCTION

1.1 Plasma Cell Development and Antibody Structure

Plasma cell are terminally differentiated B-cells, which are long-lived cells that produce high-affinity antibodies (Shapiro-Shelef and Calame, 2005). Antibodies are protein molecules that are composed of two identical heavy chains and two identical light chains. Disulfide bonds link the two heavy chains and each light chain-heavy chain heterodimer (Chiu *et al*., 2019). The two dimensional structure of an IgG antibody is summarised in Figure 1.1. Antibodies specifically bind to an antigen such as those on viruses and bacteria and are part of the immune defence.

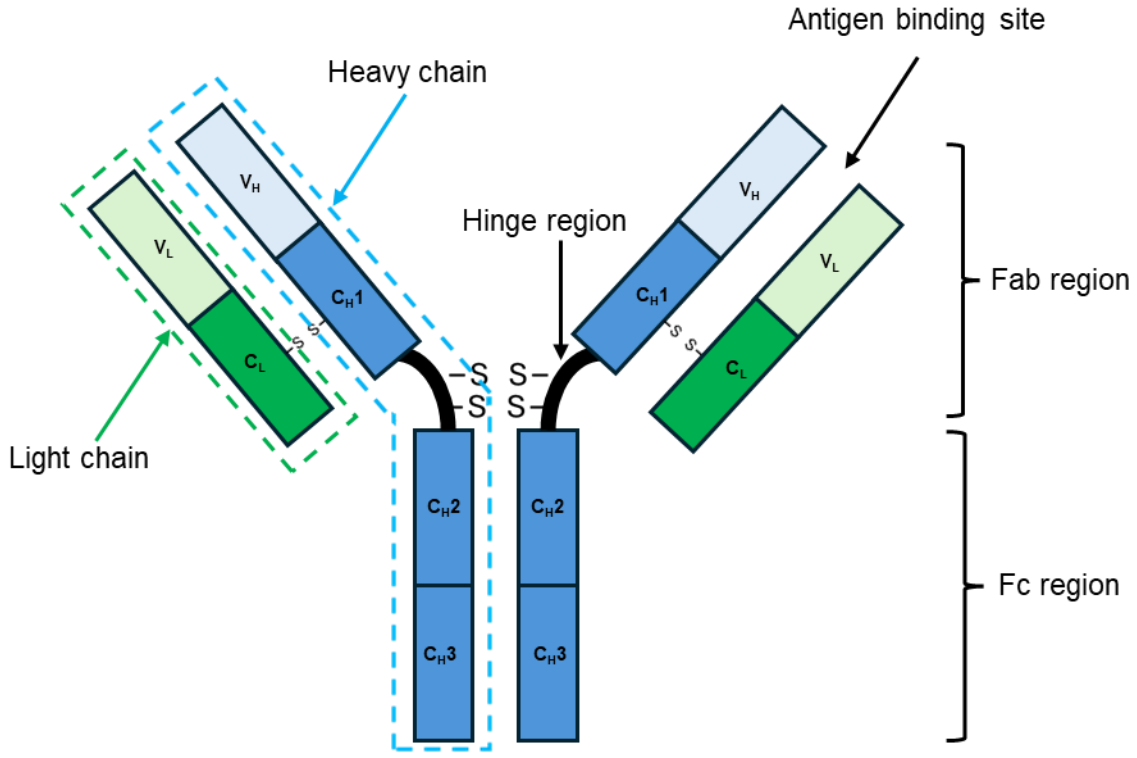


Figure 1. 1 Two-dimensional diagram of the structure of an IgG molecule
An IgG molecule is made up of two identical heavy chains and two identical light chains with a total mass of 150-160 kDa. Each heavy chain is composed of three constant domains and one variable domain, with a total mass of approximately 50-55 kDa and each light chain has a mass of approximately 22.5-25kDa (Schroeder and Cavacini, 2010).

There are five heavy chain classes – IgG, IgM, IgA, IgD and IgE-and two light chain classes – kappa and lambda. The immunoglobulin heavy chain (IgH) gene locus is on chromosome 14q32 (Croce *et al*., 1979) and immunoglobulin kappa and lambda light loci are found on chromosomes 2p11 and 22q11, respectively (McBride *et al*., 1982). Heavy chains and light chains are both composed of variable regions, which determine antibody specificity, and constant regions, which determine the antibody class and function. Both kappa and lambda light chains contain one constant domain and one variable domain. All of the heavy chains contain one variable domain but the number of constant domains differs between the classes. IgG, IgA and IgD contain three constant domains and IgM and IgE contain four constant domains. IgA and IgM antibodies have a J-chain, which enables them to form dimers and pentamers, respectively (Chiu *et al*., 2019).

In adults the process of B-cell development begins in the bone marrow where haemopoietic stem cells differentiate into common lymphoid progenitors. Commitment to the B-cell lineage occurs at initiation of the sequential rearrangement of immunoglobulin genes. The IgH variable region DNA is composed of variable (V), diversity (D) and joining (J) segments (Early *et al*., 1980) and the light chain variable regions are composed of variable and joining segments (Seidman *et al*., 1979). Lymphoid specific recombination activating gene (RAG)-1 and RAG-2 are required for the initiation of VDJ recombination (Oettinger *et al*., 1990). VDJ rearrangement occurs in a specific order- IgH D and J recombination takes places first, then IgH V rearrangement follows and the rearranged V segment is attached to the rearranged DJ segment by VDJ recombinase (Bassing *et al*., 2002).

The rearrangement of light chain V and J genes then occurs, with kappa gene rearrangements preceding lambda gene rearrangements. B-cells that have B-cell receptors with a strong affinity for self-antigens undergo apoptosis via a process of negative selection (Hartley *et al*., 1993, Sandel and Monroe, 1999). Non-self-reactive B-cells become naïve B cells which express surface IgM and circulate to the spleen where they differentiate into long-lived mature follicular B cells or marginal zone B cells (Shapiro-Shelef and Calame, 2005).

Upon antigen encounter and with follicular T helper cell support, follicular B cells undergo the germinal centre reaction, which results in the generation of high affinity antibodies through somatic hypermutation of IgH V genes and affinity maturation which occurs as the cells undergo multiple rounds of proliferation (Shapiro-Shelef and Calame, 2005). The B cells change from the generation of IgM to the generation of IgG, IgA and IgE via class switch recombination of the IgH locus, which is usually a deletional process of DNA to allow expression of an alternative downstream heavy chain gene. Both of these processes are initiated by the enzyme activation-induced cytidine deaminase (Muramatsu *et al*., 2000). During affinity maturation B cells producing high affinity antibodies are positively selected and differentiate into memory B cells and long-lived plasma cells (Shapiro-Shelef and Calame, 2005).

Errors occurring during VDJ gene re-arrangement, somatic hypermutation and class switch recombination can give rise to translocations involving the IgH locus and oncogenes, which are frequently observed in patients with plasma cell disorders (PCD) and other B cell malignancies (Walker *et al*., 2013). Translocations involving

the IgH locus on chromosome 14q32 occur during class switch recombination and are a defining initiating event for the majority of multiple myeloma tumours (Fonseca *et al*., 2003, Kaufmann *et al*., 2004).

1.2 Spectrum of PCD

PCD encompass a group of disorders which are characterised by the proliferation of a clonal population of plasma cells, which secrete monoclonal immunoglobulin or polypeptide subunits in the serum and/or urine. Patients presenting with suspected PCD undergo testing to confirm the presence and size of the monoclonal protein and to assess for end-organ damage. The results of these investigations enable the PCD to be characterised based on: the amount of monoclonal protein produced; the percentage and site/s of the clonal plasma cell population; and the presence and nature of any organ damage related to the clonal plasma cells and/or the secreted monoclonal immunoglobulin.

1.2.1 Monoclonal Gammopathy of Undetermined Significance (MGUS)

MGUS is the most common PCD, and its prevalence increases with age; 3% of individuals over the age of 50 have MGUS and this rises to 7% for individuals over 80 years of age (Kyle *et al*., 2018). MGUS is characterised by low circulating levels of monoclonal protein (<30 g/L), <10% clonal plasma cells in the bone marrow and the absence of end-organ damage related to the plasma cell clone (Rajkumar *et al*., 2014). This definition based on the percentage of plasma cells and size of the monoclonal protein does not correlate with any biological changes and there is a lack of understanding surrounding the transformational events between MGUS, smouldering multiple myeloma and multiple myeloma.

Most patients with MGUS are asymptomatic and do not require any PCD directed therapy due to the low risk of progression, which is approximately 0.5-1 % per year (Dispenzieri *et al*., 2010, Kyle *et al*., 2018). However, they do require lifelong monitoring with serial blood tests every 6-12 months to monitor for progression to the blood cancer multiple myeloma. The risk stratification models can identify a group of patients with very low risk of transformation but cannot identify high-risk MGUS well (Rajkumar *et al*., 2005).

1.2.2 Solitary Plasmacytoma

A solitary plasmacytoma is a discrete mass composed of clonal plasma cells with minimal (<10%) or no bone marrow involvement. It is a rare condition with a cumulative annual incidence of less than 1 in 100 000 (Ellington *et al*., 2021) and the median age at presentation is 60-65 years of age (Thumallapally *et al*., 2017, Sharpley *et al*., 2019b). It may present as an isolated lytic lesion of the skeleton, where it is referred to as a solitary plasmacytoma of the bone, or a discrete soft tissue mass with no contact between the neoplastic plasma cells and bone, which is referred to as an extramedullary plasmacytoma. Approximately two-thirds of plasmacytomas are solitary plasmacytomas of bone (Caers *et al*., 2018) and these are most commonly found in red marrow regions. Patients typically present with pain or local compression related symptoms (Agbuduwe *et al*., 2020, Tan *et al*., 2021, Bethel *et al*., 2022). Extramedullary plasmacytomas have been found in a wide number of anatomical sites but most commonly in the head and neck region (Thumallapally *et al*., 2017, Sharpley *et al*., 2019b, Agbuduwe *et al*., 2020).

Solitary plasmacytomas are treated with radical radiotherapy at a dose of 40-50 Gy with the aim of cure. However, the risk of progression to multiple myeloma is 30-53% even when local control is initially achieved (Jawad and Scully, 2009, Nahi *et al*., 2017, Thumallapally *et al*., 2017). Soft tissue/extramedullary plasmacytomas have a better prognosis in terms of progression to myeloma than solitary plasmacytomas of bone and can sometimes be resected surgically as an alternative treatment modality to radiotherapy depending on the location of the plasmacytoma (Thumallapally *et al*., 2017, Sharpley *et al*., 2019b).

1.2.3 Multiple Myeloma

Multiple myeloma is caused by a larger neoplastic plasma cell clone and is defined by a monoclonal protein ≥30 g/L, Bence Jones protein (BJP) ≥500 mg/24 hours and/or clonal plasma cells ≥10% on bone marrow biopsy. It is the second most common haematological malignancy with approximately 6000 new cases diagnosed each year in the United Kingdom (CRUK., 2022). The median age at diagnosis is 72 years (Raluy *et al*., 2014) and <5% patients are diagnosed below the age of 40 (Kyle *et al*., 2007).

1.2.3.1 Classification of Multiple Myeloma

Multiple myeloma is divided into smouldering multiple myeloma and symptomatic multiple myeloma based on whether there is any evidence of myeloma-related organ damage. Myeloma-related end-organ damage includes: hypercalcaemia (adjusted calcium >2.75 mmol/L); renal impairment due to myeloma (serum creatinine >177 μmol/L or creatinine clearance <40 ml/minute); anaemia (Hb <100g/L or 20g/L below the lower limit of normal); and lytic bone lesions on skeletal survey, CT or PET-CT (IMWG, 2003).

Three biomarkers of malignancy, which identify patients who have an 80% chance of developing end organ damage within the next two years, were also added to the criteria that define symptomatic myeloma in 2014. The biomarkers of malignancy are: ≥60% neoplastic plasma cells on bone marrow biopsy; ratio of the involved free light chain (FLC) to uninvolved serum FLC ≥100 (the concentration of the involved FLC (iFLC) must be ≥100 mg/L); >1 focal lesion on MRI. The combined acronym for these clinical features is SLiM-CRAB and the presence of one or more of these criteria classifies multiple myeloma as being symptomatic (Rajkumar *et al*., 2014).

Approximately 80-90% of patients with newly diagnosed multiple myeloma present at the symptomatic stage (Kyle *et al*., 2007, Ravindran *et al*., 2016, Brozova *et al*., 2017, Sidiqi *et al*., 2020). Symptoms related to bone disease and anaemia are the most common presenting symptoms and are present in over 50% of patients with newly diagnosed multiple myeloma (Mateos *et al*., 2020a, Graziani *et al*., 2020, Atkin *et al*., 2021). Renal impairment is also frequently present at diagnosis and is present in 11-47% of patients (Yadav *et al*., 2018, Ho *et al*., 2019, Graziani *et al*., 2020, Atkin *et al*., 2021, Rana *et al*., 2020, Courant *et al*., 2021). Due to the frequent occurrence of some of the presenting symptoms, such as back pain, in the general population due to much commoner causes, the diagnosis of multiple myeloma is commonly delayed and approximately one-third of patients are diagnosed following an emergency presentation (Atkin *et al*., 2021). Monitoring of patients with MGUS and smouldering multiple reduces the likelihood of patients presenting with catastrophic end-organ damage (Howell *et al*., 2017).

1.2.3.2 Pathogenesis of Multiple Myeloma

Multiple myeloma evolves as a multi-step process and it has been shown that almost all cases arise from the precursor condition MGUS (Landgren *et al*., 2009, Weiss *et al*., 2009). The early genetic changes that occur at initiation of PCD disorders include hyperdiploidy and/or translocations involving the IgH locus on chromosome 14 (Kaufmann *et al*., 2004, Bochtler *et al*., 2008, Rasche *et al*., 2017b). Translocations involving the IgH locus are found in 24-59% of patients with MGUS (Bochtler *et al*., 2008, López-Corral *et al*., 2011, Mikulasova *et al*., 2017) and 37-83% of patients with multiple myeloma (Fonseca *et al*., 2003, Bochtler *et al*., 2008, López-Corral *et al*., 2011, Mikulasova *et al*., 2017, Mao *et al*., 2020). The most common translocations involving the IgH locus are: t(4; 14) which leads to overexpression of fibroblast growth factor 3 and multiple myeloma set (Chesi *et al*., 1998b); t(11;14) which causes overexpression of cell cyclin D1 (Hoyer *et al*., 2000); t (6;14) which leads to overexpression of cyclin D3 (Shaughnessy *et al*., 2001); and t(14: 16) which results in overexpression of c-MAF (Chesi *et al*., 1998a). In 14-20% of patients with an IgH translocation the chromosomal partner is not known (Bochtler *et al*., 2008, Mao *et al*., 2020).

Clonal evolution and the development of subclonal heterogeneity occurs due to the acquisition of additional copy number aberrations, epigenetic changes such as hypermethylation and the acquisition of secondary mutations. Differential tumour microenvironmental conditions at different disease sites throughout the body lead to the development of heterogeneous subclones with varying fitness advantages (Rasche *et al*., 2017b). RAS and TP53 mutations and amplification of 1q play an important role in the process of clonal selection and are found with increasing

frequency with progression from MGUS to myeloma (Bochtler *et al*., 2008) and following anti-myeloma treatment (Misund *et al*., 2022, Vo *et al*., 2022).

Alterations in immunosuppressive cell populations, such as regulatory T-cells and myeloid-derived suppressor cells, and dysregulation of immune cells such as T-lymphocytes, NK cells and dendritic cells likely play a key role in disease progression (Schinke et al., 2022, Botta et al., 2023, Soekojo and Chng, 2022). The immune microenvironment also influences the depth and duration of response to anti-myeloma therapies (Pessoa de Magalhães et al., 2013, Coffey et al., 2023, Friedrich et al., 2023, Firestone et al., 2024).

Interactions between the myeloma cells and other cells within the tumour microenvironment and the cell-matrix also play a crucial role in the proliferation of the neoplastic plasma cells and their relative resistance to apoptosis. The cell-cell interactions protect the myeloma cells against immune cell death by promoting upregulation of immune checkpoints such as PD-L1 and inhibiting T cell activity (An *et al*., 2016, Chen *et al*., 2018a). The interaction between the myeloma cells and the bone marrow stromal cells also enhance the production of pro-survival factors such as TNF-α and growth promoting cytokines such as IL-6 and insulin-like growth factor 1 (Klein *et al*., 1989, Carter *et al*., 1990, Caligaris-Cappio *et al*., 1991, Uchiyama *et al*., 1993, Barillé *et al*., 1995, Georgii-Hemming *et al*., 1996, Chauhan *et al*., 1996, Ge and Rudikoff, 2000). Hypoxia-inducible factors promote angiogenesis and contribute to resistance to anti-myeloma therapies (Martin *et al*., 2010, Storti *et al*., 2013, Ria *et al*., 2014, Tsubaki *et al*., 2019).

Lytic bone lesions arise in myeloma due to increased osteoclast activity, which results in enhanced bone resorption. Myeloma cells express the receptor activator of nuclear factor kappa B ligand (RANKL) and induce overexpression of RANKL in bone marrow stromal cells, which leads to increased osteoclastogenesis (Abe *et al*., 2002). Myeloma cells also inhibit osteoprotegrin, which is an antagonist of RANKL, leading to an imbalance between RANKL and osetoprotegrin levels, which promotes osteoclastogenesis and activation (Croucher *et al*., 2001, Sezer *et al*., 2003). Myeloma is unique for a malignancy in that it also switches off osteoblast activity, leading to an absence of new bone formation and sclerosis on imaging (Oshima et al., 2005).

1.2.4 AL Amyloidosis

AL amyloidosis is characterised by a clonal population of plasma cells (>95% of cases) or lymphoid cells (<5% of cases) that secrete either an intact immunoglobulin or FLC only monoclonal protein with structurally abnormal light chains that misfold and form ß-pleated sheets (Sanchorawala *et al*., 2006, Palladini *et al*., 2023). AL amyloidosis is a rare haematological disorder with an annual incidence of 3-15 cases per million per year (Pinney *et al*., 2013, Quock *et al*., 2018, Kumar *et al*., 2022, Hou *et al*., 2022, Mellqvist *et al*., 2023). The incidence of AL amyloidosis increases with advancing age and the median age at diagnosis is 65-70 (Mellqvist *et al*., 2023, Palladini *et al*., 2023).

Multisystem involvement is frequently observed at diagnosis, resulting in patients presenting with a complex constellation of symptoms. The most commonly affected organs are the heart and the kidneys (Sidana *et al*., 2020b, Palladini *et al*., 2023). In

the kidney, amyloid is deposited in the glomeruli and this leads to proteinuria. In the heart amyloid is deposited within the myocardium leading to a hypertrophic heart and the degree of heart involvement is powerfully prognostic (Palladini *et al*., 2023). As a consequence of these two organs being commonly affected, signs and symptoms related to fluid overload and hypo-albuminaemia, including peripheral oedema, pleural effusions and dyspnoea, are frequently observed. Cardiac arrhythmias are often fatal and difficult to manage. Nervous system involvement may affect both the peripheral and autonomic nervous systems; peripheral nervous system involvement usually presents as sensory disturbance, whilst autonomic dysfunction typically presents as orthostatic hypotension (Wechalekar *et al*., 2015). Gastrointestinal and hepatic involvement are observed in less than 20% of patients (Lim *et al*., 2015, Sidana *et al*., 2020b) and lead to symptoms related to altered gastrointestinal mobility, gastrointestinal bleeding and hepatic enlargement, including: altered bowel habit (diarrhoea or constipation); dysphagia; right upper quadrant discomfort; early satiety; nausea and vomiting; and malaena (Wechalekar *et al*., 2015).

Owing to the invasive nature of the procedure/s required to obtain histological confirmation of organ involvement, biopsy of each involved organ is not required. Confirmation of the presence of AL amyloidosis is obtained using Congo red staining, which displays apple green birefringence in the presence of amyloid deposits. Mass spectrometry (MS) -based subtyping is employed to confirm that the amyloid deposits are of AL-amyloid subtype. Involvement of additional organ systems is then assigned based on clinical examination findings, biochemistry and imaging results once histological evidence of AL amyloid deposition has been demonstrated at a single site (Wechalekar *et al*., 2015).

1.4.1.1 Pathogenesis of AL Amyloidosis

Systemic AL amyloidosis is caused by the deposition of structurally abnormal immunoglobulin molecules which form fibrillar deposits in affected organs, leading to progressive organ dysfunction. Amyloidogenic light chains have amino acid substitutions which lead to reduced stability and promote the formation of misfolded proteins which are more aggregation prone (Hurle *et al*., 1994, Raffen *et al*., 1999, Rottenaicher *et al*., 2021). N-linked glycosylation has also been shown to increase the tendency of kappa light chains for forming amyloid fibrils (Stevens, 2000). The binding of amyloid fibrils to serum amyloid protein confers resistance to proteolysis, enabling their persistence and deposition in target organs (Tennent *et al*., 1995). The variable organ tropism of amyloidogenic light chains can be at least partially explained by the light chain variable gene of the clone, for example e.g. IGLV1-44 is associated with dominant cardiac involvement (Perfetti *et al*., 2012) and LV6-57 usage is linked to predominant renal involvement (Kourelis *et al*., 2017). Tissue factors such as extracellular matrix composition, shearing forces and the presence of metals ions are also thought to be important factors in the promotion of fibril formation (Merlini, 2017).

The clone size is typically much smaller than those seen in patients with symptomatic multiple myeloma and patients commonly present with an insidious onset of symptoms related to organ dysfunction, which has been caused by the deposition of the insoluble amyloid fibrils in affected organs (Wechalekar *et al*., 2015). There is also evidence that the amyloidogenic light chains can directly cause cardiac myocyte cell death, independent of amyloid fibril formation (Brenner *et al*., 2004).

1.2.5 Plasma Cell Leukaemia

Plasma cell leukaemia is a rare and an aggressive plasma cell neoplasm with an annual incidence of approximately 0.04 cases per 100 000 people (Sant *et al*., 2010). Plasma cell leukaemia is defined by the presence of ≥5% circulating neoplastic plasma cells in the peripheral blood. When it arises in patients with no prior history of multiple myeloma, it is described as primary plasma cell leukaemia (60-70% of cases) and in patients with a prior history of multiple it is described as secondary plasma cell leukaemia (Bladé and Kyle, 1999, Fernández de Larrea *et al*., 2021).

The median age at presentation of primary plasma cell leukaemia is 57-69 years of age (Granell *et al*., 2017, Nandakumar *et al*., 2021, Ge *et al*., 2022, Peña *et al*., 2023, Guan *et al*., 2023, Saba *et al*., 2024). Patients with primary plasma cell leukaemia commonly present with similar symptoms to those with myeloma but there is a higher incidence of thrombocytopenia and the average plasma cell infiltration in the bone marrow is higher (Fernández de Larrea *et al*., 2021, Nandakumar *et al*., 2021, Jung *et al*., 2022).

1.2.6 Conditions Associated with IgM Monoclonal Proteins

Although IgM monoclonal proteins account for 15-20% of all monoclonal proteins, IgM multiple myeloma is a rare subtype of multiple myeloma as IgM monoclonal proteins are usually secreted by lymphoplasmacytoid cells rather than plasma cells (Kyle *et al*., 2006). The most common conditions associated with IgM monoclonal proteins are MGUS, lymphoplasmacytic lymphoma, marginal zone lymphoma and chronic lymphocytic leukaemia (Kyle and Garton, 1987). The majority of patients with a symptomatic haematological malignancy and an IgM monoclonal protein have

lymphoplasmacytic lymphoma. The median age at diagnosis for patients with lymphoplasmacytic lymphoma is 73 years of age (Montesdeoca *et al*., 2023).

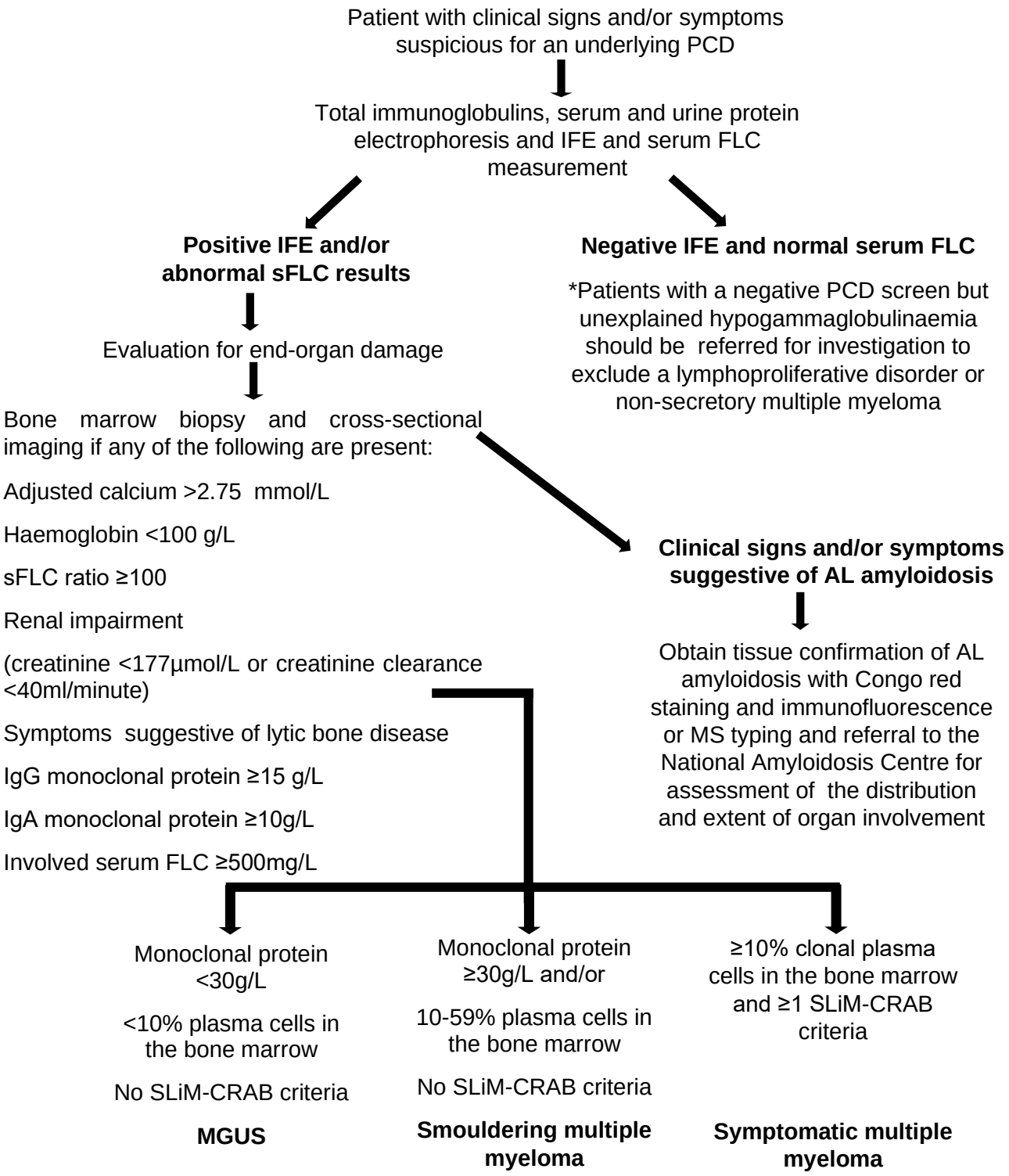
The presenting features depend on the subtype of lymphoproliferative disorder. Bulky lymphadenopathy is uncommon in lymphoplasmacytic lymphoma (Montesdeoca *et al*., 2023). Renal impairment and lytic bone disease are rare in lymphoplasmacytic lymphoma (Bhatti *et al*., 2022, Danesin *et al*., 2024, Brunner *et al*., 2024) but leukaemic involvement at presentation is more frequent than in multiple myeloma (Montesdeoca *et al*., 2023).

The mutational landscape of lymphoplasmacytic lymphoma is also distinct from that seen in multiple myeloma – most patients with lymphoplasmacytic lymphoma have a MYD88 mutation, which is a single amino acid substitution (L265P) that is activating (Treon *et al*., 2012, Xu *et al*., 2013, Varettoni *et al*., 2013, Hunter *et al*., 2014, Brunner *et al*., 2024), and approximately a quarter have subclonal CXCR4 mutations (Hunter *et al*., 2014, Brunner *et al*., 2024). Owing to the distinct pathophysiology and immunophenotype (Seegmiller *et al*., 2007), the treatment and prognosis of lymphoplasmacytic lymphoma differs from that of multiple myeloma (Schuster *et al*., 2010).

1.3 Investigation of Patients with Suspected PCD

Patients with PCD may be diagnosed at an asymptomatic stage when they are being investigated for other medical conditions that may present with similar symptoms, such as rheumatological and neurological conditions, or they may present at a symptomatic stage either as a two-week wait referral or as an emergency presentation. The

management differs depending on the nature and stage of the PCD, so a comprehensive panel of investigations is undertaken to ensure that the correct type and stage of disorder is diagnosed. The screening pathway utilised for patients with suspected PCD at University Hospitals Birmingham (UHB) NHS Foundation Trust is shown in Figure 1.2.



*all patients have their albumin: creatinine ratio and NTproBNP levels checked at diagnosis

Figure 1. 2 Screening algorithm for patients referred to UHB NHS Foundation Trust with suspected PCD

Patients are stratified according to their presenting symptoms, size of the monoclonal protein and presence or absence of end-organ damage and this guides which further investigations should be performed to investigate for a suspected a suspected PCD. The results are then used to classify the PCD.

In this pathway all patients with a detectable monoclonal protein are initially stratified according to the presence or absence of any end organ damage. Patients with evidence of end-organ damage on their blood tests (Haemoglobin <100 g/L, renal impairment due to suspected myeloma (creatinine >177 µmol/L or creatinine clearance <40ml/minute), hypercalcaemia (adjusted calcium >2.75mmol/L) or symptoms or imaging findings suggestive of lytic bone disease undergo bone marrow biopsy and cross-sectional imaging. In addition all patients with a FLC ratio of ≥100 undergo bone marrow biopsy and cross-sectional imaging. These criteria are aligned with the SLiM CRAB criteria proposed by the International Myeloma Working Group (IMWG), which are used to define patients with symptomatic myeloma.

Patients with no evidence of end organ damage on initial blood work up are stratified based on the level of the monoclonal protein detected. Patients with an a monoclonal protein <10 g/L are unlikely to have myeloma unless the FLC ratio is significantly abnormal (Heaney *et al*., 2020) and therefore do not routinely undergo bone marrow biopsy. In the UHB NHS foundation Trust guidelines the thresholds for performing a bone marrow biopsy differ for patients with IgG and IgA monoclonal proteins and this is based on the fact the average monoclonal protein level of patients presenting with IgA myeloma is lower than in patients with IgG myeloma (Heaney *et al*., 2020). The thresholds for performing bone marrow biopsies in asymptomatic individuals with IgG and IgA monoclonal proteins are in keeping with the thresholds included in the European Myeloma Network and British Society of Haematology (BSH) recommendations for the investigation of patients with MGUS (van de Donk *et al*., 2014, Stern *et al*., 2023). There are several international guidelines that do not specify

specific monoclonal protein, BJP or serum FLC ratio thresholds for proceeding to do a bone marrow biopsy and cross-sectional imaging and use the Mayo MGUS risk stratification score instead (Hillengass *et al*., 2019, Bergstrom *et al*., 2020).

In the UHB protocol a BJP threshold for performing bone marrow biopsy and cross-sectional imaging is not included as urine protein electrophoresis and urine IFE has largely been replaced by serum FLC measurements in this trust. The reliance on serum FLC measurements rather than urine protein electrophoresis in keeping with the BSH guideline on the diagnosis, investigation and initial treatment of myeloma, which state recommend that serum FLC tests should be used to evaluate monoclonal light chains rather than urine BJP (Sive *et al*., 2021).

However, several international guidelines, including the recommended investigation panel for patients with suspected myeloma from the IMWG, still include analysing urine for total protein, urine protein electrophoresis and urine IFE (Rajkumar *et al*., 2014, van de Donk *et al*., 2014, Bergstrom *et al*., 2020, Dimopoulos *et al*., 2021). This is likely due to the fact that there is a urine monoclonal protein criteria for differentiating between light chain MGUS and light chain smouldering multiple myeloma (urine monoclonal protein ≥500mg/24 hours) but no absolute level for the involved serum FLC (Rajkumar *et al*., 2014). In addition, the UHB guidance suggests an absolute level for the involved FLC level whereas other guidelines suggest FLC ratio thresholds independent of the Mayo MGUS risk stratification score, although these differ between guidelines (van de Donk *et al*., 2014, Stern *et al*., 2023).

1.3.1 Total Immunoglobulin Measurements

Immunoturbidometric and nephelometric assays both provide quantitative results for the total amounts of IgG, IgA and IgM. Immunoturbidometric assays quantitate the amount of each immunoglobulin based on the reduction in the intensity of light passing through after the addition of antibodies specific for IgG, IgA and IgM, whereas nephelometric assays quantitate immunoglobulin concentrations based on the amount of light scatter. These assays are used to identify quantitative abnormalities in the total immunoglobulin levels, however they cannot be used in isolation to identify monoclonal proteins as elevated levels of polyclonal immunoglobulins may be seen in the context of inflammatory conditions such as liver disease, infection, autoimmune disease and malignancy (Zhao *et al*., 2020). In addition, small monoclonal proteins and light chain only monoclonal proteins may be present in the absence of elevated total immunoglobulin levels (Kastritis *et al*., 2014, Batinić *et al*., 2015, Sørrig *et al*., 2017, Hussain *et al*., 2019, Landgren, 2021, Ramasamy, 2023). Whilst many of these patients may present with suppressed polyclonal immunoglobulin levels, approximately 10% of patients with light chain only myeloma patients present with no immune paresis (Kastritis *et al*., 2014, Sørrig *et al*., 2017). Overall, in a study of 99 patients with multiple myeloma by Hussain *et al*, 18% of patients had no evidence of hypo- or hypergammaglobulinaemia at presentation (Hussain *et al*., 2019). The results therefore need to be interpreted alongside the results of electrophoretic assays and serum FLC results.

1.3.2 Serum Protein Electrophoresis (SPE) and Immunofixation Electrophoresis (IFE)

SPE separates serum proteins into five fractions: albumin; alpha1; alpha2; beta; and gamma. In serum from healthy individuals the broad range of antibodies secreted

produces a Gaussian distribution in the gamma region (Figure 1.3A). The presence of a monoclonal protein leads to an area of restricted migration, which results in the presence of sharp peak above the polyclonal background (Figure 1.3B). This enables differentiation between polyclonal increases in immunoglobulin levels, which cause an increase in the total immunoglobulin levels but do not alter the Gaussian distribution shape of the electrophoretic trace (Figure 1.3C), and monoclonal proteins (Vavricka *et al*., 2009).

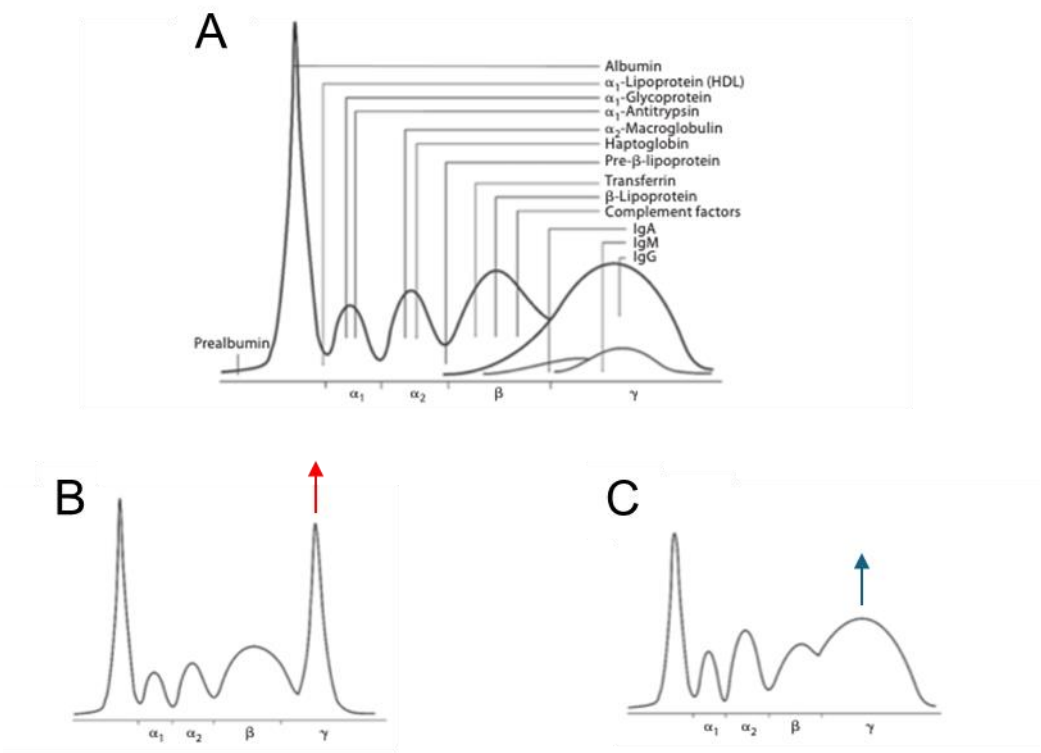


Figure 1. 3 Electrophoretic appearances of serum analysed in health and disease.

The typical appearance of serum that has undergone serum protein electrophoresis is shown in (A). (B) Shows the typical electrophoretic appearance of a monoclonal protein, which is visible as a sharp peak above the polyclonal background in the gamma region (highlighted by the red arrow). (C) Shows the electrophoretic appearances of polyclonally increased immunoglobulins in which the gaussian distribution is maintained in the gamma region (highlighted by the blue arrow).

Figure adapted from Vavricka SR et al. Serum protein electrophoresis: an underused but very useful test. Digestion 2009; 79: 203-210.

Low-level monoclonal proteins and those identified in patients with increased polyclonal immunoglobulin levels may fail to resolve into a discrete band. The arbitrary

placement of gates to quantitate low-level monoclonal proteins can result in significant inter-test variability. Due to these limitations, the IMWG uniform response criteria state that in order for the monoclonal protein to suitable to use as a biomarker of response the monoclonal protein concentration should be ≥10 g/L at baseline (Durie *et al*., 2006). In addition to the challenges in being accurately able to quantify small monoclonal proteins, 40-50% of IgA monoclonal proteins migrate in the beta-region alongside other serum proteins, such as transferrin and complement, and in these patients the monoclonal protein cannot be accurately quantified by SPE and densitometry (Visram *et al*., 2020). In this circumstance, the IMWG recommends that total IgA measurements are used to track the initial therapeutic response as utilising monoclonal protein quantitation in these patients has been shown to overestimate the depth of response (Durie *et al*., 2006, Visram *et al*., 2021).

IFE utilises antisera specific for IgG, IgA, IgM, kappa and lambda light chains in combination with electrophoretic separation. A positive IFE is indicated by a band in the region of antibody-antigen equivalence (Abraham, 2013). Serum IFE is used to confirm the presence and isotype of all newly diagnosed monoclonal proteins. Figure 1.4 shows an example serum IFE result demonstrating an IgA kappa monoclonal protein from a patient with smouldering multiple myeloma. Serum IFE also provides greater sensitivity than SPE - the limit of detection of SPE is approximately 0.5-1.0g/L and the limit of detection of serum IFE is approximately 50-100mg/L (Dispenzieri *et al*., 2009, Keren, 2012).

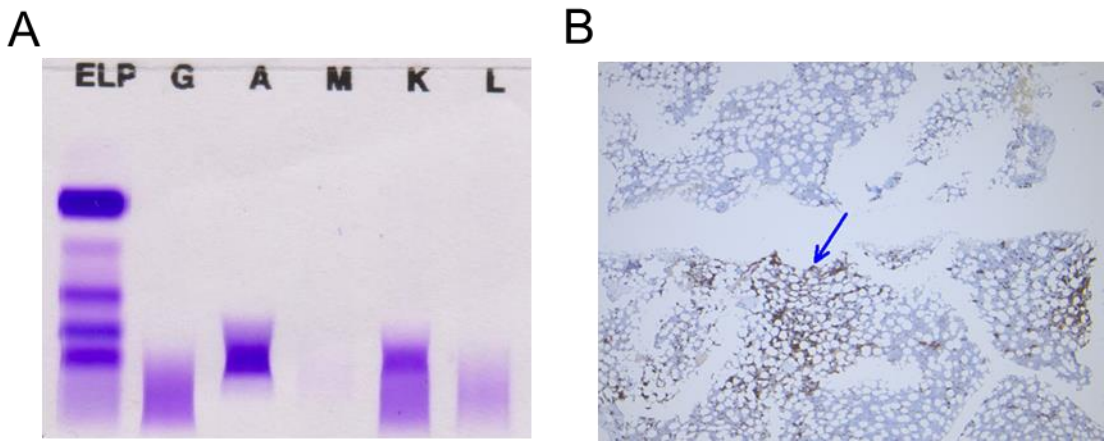


Figure 1. 4 Serum IFE confirms the presence of an IgA kappa monoclonal protein in a patient with smouldering multiple myeloma.
Distinct bands in the IgA and kappa lanes, confirming the presence of an IgA monoclonal protein in a patient with newly diagnosed smouldering multiple myeloma in (A). (B) Shows the bone marrow trephine confirming the presence of a low-level plasma cell infiltrate. The plasma cells have been stained for CD138 and are highlighted by the blue arrow. The patient provided consent for inclusion of these images in publications, including a case report.

1.3.3 Measuring Monoclonal FLC

In the absence of a clonal PCD, light chains are overproduced compared to heavy chains by approximately 40%, leading to the release of 500mg per day of excess polyclonal FLC (Waldmann *et al*., 1972, Solomon, 1985). The excess FLC are catabolised by the proximal tubular epithelial cells in the kidney within a few hours resulting in very low levels of circulating FLC in healthy individuals. However, in patients with high levels of monoclonal FLC production the re-absorptive capacity of the proximal tubular cells becomes saturated, resulting in increased levels of FLC in the urine (Basnayake *et al*., 2011, Stokes *et al*., 2016). The excess monoclonal FLC are frequently present in the serum at levels that are masked by the total light chain signals derived from the polyclonal intact immunoglobulin background (Vermeersch *et al*., 2008, Heaney *et al*., 2017). SPE and serum IFE can miss low-level FLC monoclonal proteins, in patients with conditions associated with low-level monoclonal FLC production, such as AL amyloidosis and oligo-secretory myeloma sensitivity for the detection monoclonal FLC (Dejoie *et al*., 2016, Wilhite *et al*., 2021). In a large study

of Katzman *et al*, there was no detectable monoclonal protein using serum IFE in 13.1% of patients with AL amyloidosis and 5.6% of patients with multiple myeloma at presentation (Katzmann *et al*., 2009). It is therefore essential that assays specifically designed for the detection of monoclonal FLC are incorporated into the screening panel used to investigate patients with suspected PCD to ensure FLC only secreting disease, which accounts for 15-20% of patients with multiple myeloma (Heaney *et al*., 2020), is not missed (NICE, 2016). In addition, a small proportion of patients switch from having disease which secretes both intact immunoglobulin monoclonal protein and FLC to disease that only secretes monoclonal FLC, a phenomenon referred to as 'FLC escape' (Brioli *et al*., 2014, Tacchetti *et al*., 2016, Patel *et al*., 2018).

Once the re-absorptive threshold of the proximal tubular epithelial cells has been exceeded, monoclonal FLC leak into the urine and can be identified and quantified using urine immunofixation electrophoresis and urine protein electrophoresis. The sensitivity of these techniques is enhanced by pre-concentrating the samples prior to analysis, providing a limit of detection (LOD) of approximately 10-40 mg/L (Salomo *et al*., 2002, Graziani *et al*., 2003, Viedma *et al*., 2005). In order to ensure that the level of monoclonal light chain detectable in the urine can provide a sufficiently reliable biomarker to assess response in patients with multiple myeloma, the IMWG states that the BJP concentration must be ≥200mg/24 hours at presentation (Kumar *et al*., 2016). In the study by Katzmann *et al*, the addition of urine IFE and serum IFE improved diagnostic sensitivity but there was still no detectable monoclonal in 3.3 % of patients with AL amyloidosis and 1.3% of patients with multiple myeloma at presentation (Katzmann *et al*., 2009).

Approximately 20% of patients with FLC only multiple myeloma have a BJP level <200 mg/24 hours at presentation (Heaney *et al*., 2017). In such patients, serum assays utilising antibodies specific for FLC provide greater sensitivity (Drayson M, 2001, Bradwell *et al*., 2003, Holding *et al*., 2011, Dejoie *et al*., 2016, Heaney *et al*., 2017).

Although serum FLC assays can measure FLC concentrations down to below 1 mg/L (Maisin *et al*., 2013, Shastri *et al*., 2023), the level of excess FLC production needs to sufficiently high to skew the FLC ratio at presentation and needs to be present in a sufficient concentration to be able to reliably measure serological responses. The IMWG therefore stipulates that for disease to measurable in patients undergoing treatment for symptomatic multiple myeloma using the serum FLC assays the involved FLC concentration needs to be ≥100 mg/L and the difference between the involved and uFLC needs to be ≥100 mg/L and the FLC ratio must be abnormal. In addition, the IMWG recommends that serum FLC measurements should only be used to assess response in patients with multiple myeloma when the monoclonal component in the serum and urine are not measurable by SPE (Kumar *et al*., 2016). However, in routine clinical practice serum FLC measurement is commonly assessed in place of urine protein electrophoresis and IFE as the electrophoretic assays require a 24-hour urine collection, which is impractical for many elderly frail patients. This approach is endorsed by the BSH (Sive *et al*., 2021).

Although the serum FLC assays have been widely used since their introduction, there is still a lack of consensus on the most appropriate reference ranges to use. Owing to the fact that the kidneys play a significant role in the clearance of FLC, the levels and

ranges of FLC ratio observed differ significantly between patients with normal renal function and those with varying degrees of renal impairment. Over the years, several modified reference ranges have been proposed but none have been universally adopted (Hutchison *et al*., 2008b, Molina-Andújar *et al*., 2020, Long *et al*., 2022). It is however, widely recognised that utilising the standard Freelite® reference range in individuals with severe renal impairment will result in false positive results (Hutchison *et al*., 2008b, Molina-Andújar *et al*., 2020, Long *et al*., 2022). In the IMWG guidelines on serum FLC analysis in patients with myeloma and related disorders, they do not specify a modified reference range for patients with renal impairment but acknowledge that results interpretation can be difficult in this patient cohort and suggest that tracking the iFLC level and the dFLC can be useful (Dispenzieri *et al*., 2009). The BSH guideline on the investigation and management of MGUS utilises the iStopMM renal reference range for patients with an estimated glomerular filtration rate (eGFR) <30 ml/minute/m^2 (Stern *et al*., 2023).

The iStopMM group have recently proposed that the reference ranges for patient without significant renal impairment should also be modified based on age (Einarsson Long *et al*., 2023). The standard Freelite® reference ranges and the amended reference ranges proposed by the iStopMM group are outlined in Table 1.1.

Table 1. 1 Standard and renal reference ranges for the interpretation of serum FLC results obtained using the Freelite® assay

Standard Freelite Reference Range (Katzmann *et al*., 2002)	**FLC ratio**	**Serum Free Kappa (mg/L)**	**Serum Free Lambda (mg/L)**
All patients	0.26-1.65	3.3-19.4	5.7-26.3
Modified renal reference range (Hutchison *et al*., 2008b)	**FLC ratio**		
Range derived from patients with dialysis dependent renal failure	0.37-3.1		
iStopMM renal reference ranges (Long *et al*., 2022)	**FLC ratio**	**Serum Free Kappa (mg/L)**	**Serum Free Lambda (mg/L)**
eGFR 45-59 ml/minute/m^2	0.46-2.62	7.8-83.6	7.3-65.1
eGFR 30-44 ml/minute/m^2	0.48-3.38	8.8-103.3	8.2-73.2
eGFR <30 ml/minute/m^2	0.54-3.30	11.7-265.1	12.6-150.9
iStopMM reference ranges for patients with GFR ≥60 ml/minute/m2 (Einarsson Long *et al*., 2023)	**FLC ratio**	**Serum Free Kappa (mg/L)**	**Serum Free Lambda (mg/L)**
<70 years of age	0.44-2.16	6.3-39.0	5.9-36.7
≥70 years of age	0.46-2.59	7.0-55.8	6.4-48.0

1.3.4 Bone Marrow Biopsy

In patients with a monoclonal protein and evidence of end-organ damage a bone marrow biopsy is performed to confirm a diagnosis. At UHB, patients with an IgG monoclonal protein ≥15 g/L, IgA monoclonal protein ≥10 g/L, and iFLC ≥500mg/L also undergo bone marrow sampling to exclude a diagnosis of multiple myeloma rather than a bulky MGUS. There is a significant difference in annual risk of progression to symptomatic multiple myeloma between MGUS and smouldering multiple myeloma, particularly in the first five years after diagnosis -1% per year for MGUS versus 10% year smouldering multiple myeloma (Kyle *et al*., 2010). It is therefore important to differentiate between the two to enable haematologists to counsel patients appropriately and also to guide the appropriate follow-up frequency.

The size of the plasma cell infiltrate is assessed in three ways on the bone marrow biopsy: morphologically on the bone marrow aspirate; immunophenotypically by flow cytometric analysis of the bone marrow aspirate; and with immunohistochemistry on the bone marrow trephine sample. Multicolour flow cytometry and immunohistochemistry are also used to determine if the plasma cells have a neoplastic phenotype based on the pattern of cell surface marker expression, including: weak or absent CD45 expression; strong CD56 expression; CD20 expression; and/or loss of CD19 expression (Menter *et al*., 2017). The presence of cytoplasmic light chain restriction is used to confirm the presence of clonal plasma cells.

1.3.5 Imaging

Imaging forms a crucial investigation in the initial identification of patients with multiple myeloma and in the staging of PCD. Conventional radiography provides a low-cost and easily accessible methodology for the identification of large lytic lesions (Mian and McCurdy, 2020). However, it has low sensitivity compared to cross-sectional imaging and is not able to detect extraosseous lesions. The IMWG therefore recommend that cross-sectional imaging with either whole body CT, PET-CT or whole body MRI should be performed in all patients where funding and facilities are available and PET-CT and whole body MRI are preferable to whole body CT scans (Chantry *et al*., 2017, Hillengass *et al*., 2019).

1.3.6 Laboratory Analysis for End-organ Damage

At initial screening and each follow-up visit a series of haematological and biochemical tests are performed to exclude the presence of end-organ damage: the full blood count is a measured to identify anaemic patients; serum creatinine and eGFR are checked

to exclude the development of acute kidney injury (AKI); and serum calcium is evaluated as myelomatous bone disease can lead to hypercalcaemia.

In addition, patients suspected of having AL amyloidosis should have biochemical test to exclude cardiac and renal involvement tested at presentation. Troponin T and N-terminal pro B-type natriuretic peptide (NT-proBNP) levels are used as screening tools to identify and stage cardiac involvement. Serum albumin and albumin: creatinine ratio (ACR) assessment are used to identify renal involvement, which typically presents with nephrotic syndrome (Sive *et al*., 2021).

1.4 Treatment of Plasma Cell Disorders

1.4.1 The Systemic Treatment of Newly Diagnosed Symptomatic Multiple Myeloma

Symptomatic multiple myeloma requires systemic treatment to prevent the development of further end-organ damage and to improve survival. The type and intensity of treatment given to patients with symptomatic multiple myeloma is determined by patient age, fitness, co-morbidities and patient choice. Patients initially receive an induction phase of chemotherapy to de-bulk the disease and prevent further end-organ damage. Induction chemotherapy typically involves giving between one and three drugs, which kill myeloma cells with differing mechanisms, in combination with corticosteroids. The mechanisms of action of the commonly used drugs in first-line myeloma regimens are outlined below.

1.4.1.1 Anti-CD38 Targeting Monoclonal Antibodies

Daratumumab was the first monoclonal antibody targeting CD38, which is highly expressed on plasma cells (Chan *et al*., 1990, Leo *et al*., 1992, de Weers *et al*., 2011), approved for use in multiple myeloma. Daratumumab kills myeloma cells via a variety of immune-mediated mechanisms including complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity, antibody-mediated cellular phagocytosis and apoptosis (de Weers *et al*., 2011, Overdijk *et al*., 2015, Overdijk *et al*., 2016). In addition, daratumumab has immunomodulatory properties- treatment with daratumumab leads to reduced numbers of CD38+ immunosuppressive cells, prevents upregulation of programmed cell death ligand 1 on antigen presenting cells and induces expansion of T-helper cells and cytotoxic T cells (Krejcik *et al*., 2016, Stocker *et al*., 2020).

Isatuximab is an alternative anti-CD38 targeting monoclonal antibody, with a similar mechanism of action to daratumumab, which is being explored in the upfront setting in several trials, including the UK-based RADAR trial (Royle *et al*., 2022).

1.4.1.2 Immunomodulatory Drugs (IMiDs)

There are currently three IMiDS in routine clinical use – thalidomide, lenalidomide and pomalidomide. The IMiDS exert their anti-myeloma activity both through direct anti-tumour effects and also by enhancing the immune system's ability to exert an anti-myeloma response (Mitsiades *et al*., 2002, Görgün *et al*., 2010). The IMiDs bind to the E3 ligase protein cereblon, which leads to the ubiquintination and proteasomal degradation of the Ikaros family zinc finger (IZKF) proteins IZKF1 and IZKF3 and downregulation of MYC and interferon regulator 4 expression resulting in cell cycle

arrest and cell death after prolonged exposure (Lopez-Girona *et al*., 2011, Zhu *et al*., 2011, Lu *et al*., 2014, Krönke *et al*., 2014). The IMiDS directly induce apoptosis of myeloma cells via caspase-8 activation (Mitsiades *et al*., 2002).

The IMiDS enhance anti-myeloma immune responses through a variety of mechanisms including: inducing the expression of co-stimulatory molecules (Görgün *et al*., 2010); reducing inhibitory immune cells (Görgün *et al*., 2010); enhancing proliferation, activation and cytotoxicity of immune effector cells (Davies *et al*., 2001, Görgün *et al*., 2010, Costa *et al*., 2017, Hideshima *et al*., 2021); increasing tumour antigen uptake by dendritic cells and efficiency of dendritic cell antigen presentation (Henry *et al*., 2013) and inducing IL-2 and interferon-γ production (Krönke *et al*., 2014). IMiDs also block myeloma cell interactions with bone marrow stromal cells and inhibit angiogenesis (Dredge *et al*., 2005). The action of the IMiDs are complex and the relative importance of each mode of action is poorly understood.

1.4.1.3 Proteasome Inhibitors

There are three proteasome inhibitors approved for clinical use in patients with multiple myeloma-bortezomib, carfilzomib and ixazomib. Bortezomib, which is a first generation proteasome inhibitor, is the most commonly used proteasome inhibitor in the first-line setting (Rajkumar, 2024). Bortezomib inhibits proteasomal degradation of IKappaB, which results in stabilisation of nuclear factor kappa-b (NFKB) and prevents its translocation to the nucleus (Ma *et al*., 2003). This leads to the inhibition of myeloma cell proliferation by preventing the upregulation of IL-6 secretion (Hideshima *et al*., 2001, Roccaro *et al*., 2006). The inhibition of myeloma cell binding to bone marrow stromal cells leads to a reduction in the secretion of angiogenic

factors, such as vascular endothelial growth factor, by the bone marrow stromal cells (LeBlanc *et al*., 2002, Roccaro *et al*., 2006).

Bortezomib induces apoptosis of myeloma cells through caspase activation, induction of p53 (LeBlanc *et al*., 2002, Hideshima *et al*., 2003) and enhancement of immune-mediated cell lysis (Shi *et al*., 2008).

Bortezomib synergises with other chemotherapeutic agents used in myeloma through a variety of mechanisms (Hideshima *et al*., 2001). Bortezomib inhibits the binding of the neoplastic plasma cells to bone marrow stromal cells, which helps overcome cell-adhesion mediated drug resistance and helps sensitise the myeloma cells to other chemotherapeutic agents (Mitsiades *et al*., 2003). It restores sensitivity to DNA-damaging chemotherapeutic agents by inhibiting genotoxic stress response pathways (Mitsiades *et al*., 2003) and bortezomib-induced apoptosis is also enhanced by IMiDs (Mitsiades *et al*., 2002).

1.4.1.4 Corticosteroids

Corticosteroids have formed a key part of anti-myeloma therapy for several decades. Dexamethasone is the most commonly used corticosteroid in myeloma but prednisolone is also used in some regimens utilised in frailer patients (San Miguel *et al*., 2008, Mateos *et al*., 2018, Facon *et al*., 2019b). Corticosteroids induce apoptosis of myeloma cells through suppressing IL-6 production via inhibition of the transcription factor NFKB (Smith *et al*., 1998, Feinman *et al*., 1999, Chauhan *et al*., 2002, Rosenberg, 2023). Dexamethasone has also been shown to contribute to apoptosis

of myeloma cells through the activation of Related Adhesion Focal Tyrosine Kinase (Chauhan *et al*., 1999), down-regulation of anti-apoptotic genes such as Bcl-xL and suppression of the expression of oncogenes such as c-myc (Chauhan *et al*., 2002). Dexamethasone treatment leads to myeloma cell death by apoptosis and autophagy (Grandér *et al*., 2009).

Dexamethasone has also been shown to synergise with IMiDs by enhancing their immunomodulatory effects (Kim *et al*., 2017) and with proteasome inhibitors (Hideshima *et al*., 2001).

1.4.1.5 Alkylating Agents

Alkylating agents such as cyclophosphamide and melphalan, induce myeloma cell death by cross-linking the two DNA strands leading to an arrest in DNA replication and cell death (Poczta *et al*., 2021). Treatment with alkylating agents has also been shown to induce immunomodulatory effects that can lead to immunogenic cell death. Treatment with alkylating agents has been shown to cause depletion of regulatory T cells (Lutsiak *et al*., 2005, Zitvogel *et al*., 2008, Tongu *et al*., 2013), improved natural killer cell function (Swan *et al*., 2020), enhance tumour antigen uptake by dendritic cells (Lu *et al*., 2015) and activation of CD8+T cells (Lu *et al*., 2015)

1.4.1.6 Chemotherapy Regimens Used for the Treatment of Patients with Newly Diagnosed Symptomatic Multiple Myeloma who are Fit Enough for an ASCT

In younger, fitter patients who are considered fit to proceed enough proceed to high dose melphalan conditioned autologous stem cell transplant (ASCT), induction

chemotherapy is typically given as triplet or quadruplet combinations, which include: a proteasome inhibitor (bortezomib, ixazomib or carfilzomib); IMiDs (thalidomide, lenalidomide or pomalidomide); dexamethasone; and for most recently approved regimens therapeutic monoclonal antibodies (daratumumab or isatuximab). These multi-agent regimens are highly effective and have led to dramatic improvements in the progression free survival (PFS) and overall survival (OS) of patients with multiple myeloma over the last 20 years. The induction regimens that are approved and commonly used for transplant-eligible patients with newly diagnosed symptomatic multiple myeloma are listed in Table 1.2. Transplant-eligible patients then proceed to receiving high-dose melphalan followed by ASCT. Response may be further consolidated with further combination chemotherapy, similar to that given during induction, or patients may proceed directly to maintenance therapy.

Table 1. 2 Treatment regimens used to treat patients with newly diagnosed multiple myeloma who are fit for ASCT

Treatment	Response Rate	Survival Outcomes
VTD with ASCT (Tacchetti *et al*., 2020, Schütz *et al*., 2020, Moreau *et al*., 2019)	62-82.4% ≥VGPR 26% ≥CR	10 year PFS 34% 10 year OS 60%
VRD with ASCT (Attal *et al*., 2017, Voorhees *et al*., 2023, Byun *et al*., 2023)	88% ≥VGPR 59-68.6% ≥CR	Median PFS 50 months 8 year OS 62.2%
KRd with ASCT (Jasielec *et al*., 2020)	91% ≥VGPR 79% ≥CR	5 year PFS 72% 5 year OS 84%
Dara-RVD with ASCT (Voorhees *et al*., 2020, Voorhees *et al*., 2023)	99% ORR 62.6% sCR	4 year PFS 87.2%
Dara-VTD with ASCT (Moreau *et al*., 2019, Byun *et al*., 2023, Moreau *et al*., 2021)	83% ≥VGPR 39-90.5% ≥CR	Median PFS 46.7 months

VGPR very good partial response; CR complete response; ORR overall response rate; and sCR stringent complete response. VTD velcade, thalidomide and dexamethasone; VRD velcade, lenalidomide and dexamethasone; KRd carfilzomib, lenalidomide and dexamethasone; Dara-RVD daratumumab, lenalidomide, bortezomib and dexamethasone; and Dara-VTD daratumumab, bortezomib, thalidomide and dexamethasone.

1.4.1.7 Chemotherapy Regimens Used for the Treatment of Patients with Newly Diagnosed Symptomatic Multiple Myeloma who are Not Fit Enough for an ASCT

For older patients, typically those over the age of 70-75 years of age, and patients with multiple co-morbidities ASCT is associated with a higher risk of severe morbidity and transplant-related mortality and is therefore not routinely performed. This patient group represents 70-75% of patients with multiple myeloma. Depending on patient fitness, treatment is usually delivered as doublet or triplet combinations of: IMiDS; proteasome inhibitors; alkylating agents; corticosteroids (dexamethasone and prednisolone); and therapeutic monoclonal antibodies. The induction regimens that are approved and in current use for transplant-ineligible patients are listed in Table 1.3.

Table 1. 3 Treatments used to treat transplant-ineligible patients with newly diagnosed multiple myeloma.

Treatment	Response Rate	Survival Outcomes
Rd (continuos) (Bahlis *et al*., 2020, Lee *et al*., 2023, Mian *et al*., 2023, Durie *et al*., 2020, Facon *et al*., 2018, Facon *et al*., 2019a)	44.0-62.9% ≥VGPR 12.1-28.4% ≥CR	Median PFS 20.0-31.9 months Median OS 59.1 -100.0 months
RVD (Durie *et al*., 2017, O'Donnell *et al*., 2018, Kumar *et al*., 2020)	43.5-66.0% ≥VGPR 15.0-44.0% ≥CR	Median PFS 34.4-43.0 months Median OS 75 months
VMP (Mateos *et al*., 2018, Facon *et al*., 2019b, San Miguel *et al*., 2008, San Miguel *et al*., 2013)	50% ≥VGPR 23.1-30.0% ≥CR	Median PFS 19.3-22.1 months Median OS 56.4 months
VCD (Mollee *et al*., 2024, Kumar *et al*., 2012b)	28-41% ≥VGPR 4-22% ≥CR	Median PFS 16.8 months Median OS 58.7 months
Dara-VMP (Mateos *et al*., 2018, Mateos *et al*., 2020b)	71.1% ≥VGPR 42.6% ≥CR	Median PFS 36.4 months 3 year OS 78%
Dara-Rd (Facon *et al*., 2019a, Facon *et al*., 2021)	79.3% ≥VGPR 47.6% ≥CR	60 month PFS 52.5% 60 month OS 66.3%

Rd lenalidomide and dexamethasone; RVD lenalidomide, bortezomib and dexamethasone; VMP bortezomib, melphalan and prednisolone; VCD bortezomib, cyclophosphamide and dexamethasone; Dara-VMP daratumumab, bortezomib, melphalan and prednisolone; and Dara-Rd daratumumab, lenalidomide and dexamethasone.

Dose reductions are required in more elderly, frail patients and current trials, such as the Myeloma XIV trial, are aiming to develop more objective assessment tools to assist with this (Coulson *et al*., 2022).

1.4.2 Supportive Care for Patients Receiving Systemic Anti-myeloma Therapy

Infection is a significant contributor to the early mortality risk in multiple myeloma (Augustson *et al*., 2005, Atkin *et al*., 2021) and is not typically associated with

neutropenia. The administration of prophylactic levofloxacin for the first 12 weeks of anti-myeloma therapy was shown to reduce the risk of febrile episodes and death in the TEAMM trial (Drayson *et al*., 2019). The TEAMM trial also reported that although the risk of *Pneumonocystis jirovecii* infection is low in patients with newly diagnosed symptomatic multiple myeloma, the use of prophylactic co-trimoxazole reduced the risk of febrile episodes and death. However, routine administration of prophylactic co-trimoxazole is not uniformly employed. Prophylactic acyclovir is recommended for patients receiving proteasome inhibitor-based therapy, patients with a history of recurrent herpetic infections and post ASCT (Raje *et al*., 2022).

Administration of intravenous bisphosphonates is recommended for all patients with newly diagnosed symptomatic myeloma to reduce the risk of skeletal events and reduce the risk of developing malignant hypercalcaemia (Snowden *et al*., 2011, Mhaskar *et al*., 2017, Terpos *et al*., 2021). Four weekly zolendronic acid is the preferred bisphosphonate as it is the only bisphosphonate that has been shown to confer a survival benefit compared to oral sodium clodronate (Morgan *et al*., 2011, Mhaskar *et al*., 2017). Vitamin D deficiency is highly prevalent in patients with multiple myeloma (Yellapragada *et al*., 2020), so calcium and vitamin D supplementation is given alongside bisphosphonate treatment to prevent the development of severe hypocalcaemia and maximise efficacy of the bisphosphonate treatment.

Combination treatment with IMiDS and corticosteroids are associated with high rates of venous thromboembolic disease (Bradbury *et al*., 2020). Routine thromboprophylaxis with prophylactic dose low molecular-weight heparin or direct oral

anticoagulants should therefore be given to patients receiving chemotherapy regimens containing an IMiD with high-dose dexamethasone (Palumbo *et al*., 2008).

1.4.3 Treatment of Patients with Systemic AL Amyloidosis

90-95% of patients with AL amyloidosis have a clonal plasma cell population, which can be treated using plasma cell directed therapies. Due to the relative frailty of most patients with AL amyloidosis standard regimens used to treat multiple myeloma are poorly tolerated (Kastritis *et al*., 2019). Combination treatment with proteasome inhibitors and IMiDs are not frequently employed due to the risk of exacerbating symptoms related to peripheral neuropathy and autonomic dysfunction further.

The regimens that are commonly used to treat patients with systemic AL amyloidosis and their published response rates are shown in Table 1.4. A modified and attenuated version of VCD has been the most commonly employed first-line chemotherapy regimen in patients treated for systemic AL amyloidosis in the United Kingdom (Wechalekar *et al*., 2015). Recently, the addition of daratumumab to VCD has been shown to improve haematological and organ responses and reduce the risk of disease progression (Kastritis *et al*., 2021c) and has been approved by NICE on 27th March 2024 (NICE, 2024). In patients with significant peripheral neuropathy, alternative regimens such as lenalidomide and dexamethasone or melphalan and dexamethasone may be employed to avoid the risk of bortezomib causing additional neuropathic symptoms (Wechalekar *et al*., 2015). In the 5-10% of patients who have AL amyloidosis in associated with an IgM monoclonal protein, the amyloidogenic FLC are typically secreted by a B-cell clone and B-cell directed therapies, such as bendamustine with rituximab (BR), and rituximab, cyclophosphamide and

dexamethasone (CDR) are employed. AL amyloidosis associated with IgM monoclonal proteins has a worse prognosis due to the lack of complete responses in Waldenstrom's macroglobulinaemia and its precursor MGUS state with a persistence of amyloidogenic light chain production despite treatment (Sidana *et al*., 2020a).

Table 1. 4 Regimens Used to Treat Newly Diagnosed Systemic AL Amyloidosis

Regimen	Haematological Response Rate	Outcomes
Dara-VCD (Kastritis *et al*., 2021c)	53% CR	41.5% cardiac response rate 53% renal response rate
VCD (Kastritis et al., 2021c, Palladini et al., 2015)	19-23% CR	17-22% cardiac response rate 24-25% renal response rate
Mdex (Kastritis *et al*., 2020)	20% CR	28% cardiac response rate 43% renal response rate
BMDex (Kastritis *et al*., 2020)	23% CR	38% cardiac response rate 44% renal response rate
Rd (Sanchorawala *et al*., 2007)	29% CR	41% had ≥50% reduction in proteinuria

Dara-VCD daratumumab, bortezomib, cyclophosphamide and dexamethasone; VCD bortezomib, cyclophosphamide and dexamethasone; Mdex oral melphalan and dexamethasone; BMDex bortezomib, melphalan and dexamethasone; and Rd lenalidomide and dexamethasone.

High-dose melphalan conditioned ASCT in patients with AL amyloidosis is associated with a much higher treatment related morbidity and mortality compared to in patients with multiple myeloma. However, with improved patient selection and avoidance of clinical features associated with increased transplant related mortality, including advanced cardiac disease, refractory arrhythmias, history of gastrointestinal bleeding, severe orthostatic hypotension and symptomatic pleural effusions, deep and

prolonged haematological remissions can be achieved (Dispenzieri *et al*., 2001, Nguyen *et al*., 2018a).

Historically there was no established role for routine indefinite maintenance therapy until disease progression in patients with systemic amyloidosis (Wechalekar *et al*., 2022, Sanchorawala *et al*., 2022). However, the recently approved regimen Dara-VCD does include a maintenance phase of daratumumab, which must be stopped after a maximum of 24 cycles or earlier if disease progression occurs (NICE, 2024). In patients who have achieved ≥VGPR at the end of first-line line treatment, disease status is monitored 1-3 monthly. Patients with a suboptimal response to first-line treatment, typically commence second-line treatment regimens with an IMiD in combination with dexamethasone (Wechalekar *et al*., 2015).

1.4.3.1 Supportive Care for Patients undergoing Systemic Treatment for AL Amyloidosis

Patients being treated for systemic AL amyloidosis require a multidisciplinary team approach to manage amyloid-related organ dysfunction and minimise the risk of symptomatic deterioration during therapy. Prophylactic anti-infective regimens similar to those used in multiple myeloma patients are employed in patients undergoing treatment for systemic disease.

Advanced cardiac disease remains the leading cause of early mortality and may cause intractable atrial or ventricular arrythmias and/or severe ventricular dysfunction (Sayed *et al*., 2015). Amiodarone is the main pharmacological treatment used for the treatment of dysrhythmias. Diuretics form the cornerstone of the management of

volume overload due to congestive cardiac failure. Attenuated doses of dexamethasone compared to those used in multiple myeloma are employed to avoid iatrogenic exacerbation of fluid overload problems. Younger fit patients with advanced isolated cardiac disease may undergo heart transplantation in addition to clonally directed therapy (Sanchorawala *et al*., 2022).

Renal involvement is commonly associated with volume overload due to hypo-albuminaemia. The resultant oedema is managed with loop-diuretics and aldosterone antagonists, such as spironolactone, in collaboration with fluid and salt restriction. Patients with nephrotic syndrome are at increased risk of accelerated atherosclerotic disease, so modifiable risk factors such as smoking should be addressed. Patients with severe nephrotic syndrome are at increased risk of thrombosis due to secondary antithrombin deficiency and should have a risk benefit analysis to evaluate their need for prophylactic anticoagulation.

The supportive measures employed for other commonly encountered complications of AL amyloidosis are summarised in Table 1.5.

Table 1. 5 Supportive care measures for commonly encountered symptoms in patients with AL amyloidosis

Symptom	Interventions
Hypotension	Graduated compression stockings Postural training Midodrine
Peripheral neuropathy	Gabapentin or pregabalin Selective serotonin reuptake inhibitors
Malnutrition	Dietician input
Constipation	Prokinetics such as metoclopramide Stool softeners e.g. sodium docusate
Diarrhoea	Anti-motility drugs e.g. loperamide
Low mood and anxiety	Clinical nurse specialist support Psychology input Social worker input

1.5 Prognostic Factors in Patients with PCD

1.5.1 Prognostic Factors in Patients with Symptomatic Myeloma

A large number of prognostic factors, which reflect patient fitness, the biology of the neoplastic plasma cell clone and the responsiveness of the neoplastic cells to anti-myeloma therapy have been identified. Patient age and performance status have also been demonstrated to be important prognostic factors as they impact the intensity of treatment that can be delivered and also the reserve to overcome treatment-related complications such as infections (Palumbo *et al*., 2015b, Usmani *et al*., 2018, Cook *et al*., 2019, Pawlyn *et al*., 2020, Facon *et al*., 2020). The prognostic factors in multiple myeloma are summarised in Table 1.6 and discussed below.

Table 1. 6 Prognostic Factors in Patients with Newly Diagnosed Symptomatic Multiple Myeloma

Factor	Utilisation in Clinical Practice
Age and frailty (Palumbo *et al*., 2015b, Cook *et al*., 2019, Facon *et al*., 2020, Sive *et al*., 2021)	Used in addition to performance status and comorbidities to determine suitability for ASCT and guide the need for dose reductions of induction chemotherapy
IgA monoclonal protein (Usmani *et al*., 2018)	Not routinely used as a risk stratification marker for patient counselling and not incorporated into the risk stratification scores in routine clinical use
Elevated beta-2-microglobulin (Palumbo *et al*., 2015a)	Used in commonly used risk stratification models such as the ISS and R-ISS
Low albumin (Palumbo *et al*., 2015a)	Used in commonly used risk stratification models such as the ISS and R-ISS
Circulating plasma cells (Fernández de Larrea *et al*., 2021)	Used to differentiate between multiple myeloma and plasma cell leukaemia
Severe AKI requiring dialysis (Dimopoulos *et al*., 2023)	Informs treatment choice and frequently impacts clinical trial eligibility. Velcade-based regimens with twice weekly rather than once weekly dosing are recommended for patients with severe myeloma-induced impairment.
Presence of Extramedullary Disease (Usmani *et al*., 2012)	High risk feature which may inform patient counselling re prognosis
High risk cytogenetic abnormalities (Palumbo *et al*., 2015a)	Used in commonly used risk stratification models such as the R-ISS
Gene expression profiling (van Beers *et al*., 2017, Dimopoulos *et al*., 2021, Kaiser *et al*., 2023)	Used in some clinical trials as a risk stratification tool but not in routine clinical use
Response to treatment (Lahuerta et al., 2017, Usmani et al., 2018, Costa et al., 2021a, Royle et al., 2022, Derman et al., 2022)	Used in ongoing counselling discussions with patients Being explored in multiple clinical trials as a tool which may help inform optimal maintenance treatment combinations and duration

ISS: International Staging System, R-ISS: Revised International Staging System

Beta-2-microglobulin and albumin are both routinely measured at diagnosis (Cassuto *et al*., 1978, Greipp *et al*., 2005). Beta-2-microglobulin provides a biomarker, which is reflective of both tumour mass and renal function, and elevated levels are associated with shorter OS (Greipp *et al*., 2005). Serum albumin levels are inversely correlated with IL-6 levels (Kim *et al*., 2010) and may also be reflective of the overall performance

status of patients (Sullivan, 2001). These two biomarkers are combined to classify patients into three prognostic groups using the International Staging System (ISS) (Greipp *et al*., 2005).

A revised version of the ISS (R-ISS), incorporating three high-risk cytogenetic abnormalities (del(17p), t(4;14) and t(14;16)) and lactate dehydrogenase levels (LDH) was proposed by the IMWG in 2015 (Palumbo *et al*., 2015a). Elevated LDH levels reflect the presence of high tumour mass, in particular extramedullary disease, and/or a high-proliferative index of the neoplastic plasma cell clone. Although the R-ISS provides improved prognostic accuracy (Palumbo *et al*., 2015a), it does not incorporate 1q gain/amplification or differentiate between patients with a single versus multiple high-risk cytogenetic abnormalities, which have both been demonstrated to be adverse prognostic factors (Costa et al., 2021a, D'Agostino et al., 2022, Panopoulou et al., 2023). The ISS, R-ISS and second revision of the ISS (R2-ISS) are summarised in Table 1.7.

Table 1. 7 The ISS, RISS and R2-ISS Risk scores

ISS (Sidana *et al*., 2020a)	**Criteria**	**Median OS***
ISS 1	B2M <3.5 mg/L and albumin ≥35 g/L	66.0
ISS 2	B2M <3.5 mg/L and albumin <35 g/L or B2M 3.5-5.5mg/L	44.0
ISS 3	B2M >5.5. mg/L	29.0
R-ISS (Palumbo *et al*., 2015a)	**Criteria**	**5 year OS (%)**
R-ISS 1	ISS 1, normal LDH and standard risk cytogenetic abnormalities by iFISH	82.0
R-ISS 2	Not R-ISS 1 or R-ISS 3	62.0
R-ISS 3	B2M ≥5.5mg/L and either increased LDH or high risk cytogenetic abnormality by iFISH **	40.0
R2-ISS Risk Features (D'Agostino *et al*., 2022)	**Risk Score**	
ISS 2	1	
ISS 3	1.5	
del (17p)	1	
elevated LDH	1	
t (4;14)	1	
1q+	0.5	
R2-ISS	**Cumulative Risk Score**	**Median OS (months)**
R2-ISS 1	0	91.0
R2-ISS 2	0.5-1.0	66.0
R2-ISS 3	1.5-2.5	52.0
R2-ISS 4	3.0-5.0	22.0

**The survival times for the ISS were calculated before immunomodulatory drugs, proteasome inhibitors and anti-CD38 monoclonal antibodies were in widespread use*

*** High risk cytogenetic abnormalities by iFISH in the R-ISS model include del(17p), t(4;14) and t (14;16).*

At present neither the R-ISS or the second revision of the ISS are universally employed in routine clinical practice in the United Kingdom as fluorescence in situ hybridization (FISH) is relatively high-cost test and outside the context of clinical trials high-risk disease the results do not alter the initial management of patients treated in the United Kingdom. However, as the results provide useful prognostic information and therefore assist in the counselling of patients with newly diagnosed myeloma the BSH recommend that FISH testing (recommended minimum panel t(4;14), t(14;16), 17p-, 1q+ and +1p), beta-2-microglobulin, LDH and albumin should be assessed in all patients (Sive *et al*., 2021). The IMWG and EHA/ESMO guidelines also recommend that FISH testing is performed in all patients with newly diagnosed multiple myeloma (Chng *et al*., 2014, Dimopoulos *et al*., 2021) to enable calculation of the R-ISS risk score, although the minimum FISH panel recommended by the IMWG is smaller (Chng *et al*., 2014).

Therapeutic response, both in terms of depth and duration, are powerful prognostic indicators (Usmani *et al*., 2018). Progression of disease within 18-24 months, even in the absence of high-risk features at presentation, is associated with worse OS (Jimenez-Zepeda *et al*., 2015, Kumar *et al*., 2018, Maffini *et al*., 2019, Corre *et al*., 2020, Bygrave *et al*., 2021). Early progression is therefore used to define phenotypically high-risk multiple myeloma.

The presence of circulating plasma cells at diagnosis in patients with multiple myeloma is an adverse prognostic factor and their presence has been shown to correlate with other adverse prognostic factors, including: elevated beta-2-microglobulin levels; ISS

stage; and renal impairment (Usmani *et al*., 2012, Wang *et al*., 2019). Dingli *et al* also reported that the presence of circulating neoplastic plasma cells prior to ASCT was predictive of early relapse post ASCT (Dingli *et al*., 2006).

Lastly, the presence of extra-medullary disease is an adverse prognostic factor, which is best identified on PET-CT (Varettoni *et al*., 2010, Usmani *et al*., 2012, Zamagni *et al*., 2015, Alonso *et al*., 2019, Maffini *et al*., 2019). The neoplastic plasma cells in extra-medullary lesions tend to have a higher proliferative index (Rasche *et al*., 2012) and there is a higher incidence of high-risk cytogenetic abnormalities in patients with extramedullary disease (Usmani *et al*., 2012).

1.5.2 Prognostic Factors in Patients with AL Amyloidosis

The pattern and extent of organ involvement are both important prognostic factors in patients with systemic AL amyloidosis. High FLC levels and bone marrow plasma cell infiltration ≥10% are associated with more severe and widespread organ involvement, particularly cardiac involvement, and are associated with poorer outcomes (Gustine *et al*., 2022).

Approximately 30-40% of patients with systemic AL amyloidosis die within 12 months of diagnosis and a significant proportion of these deaths are due to cardiac disease (D'Souza *et al*., 2021). Markers of the severity of cardiac involvement are therefore highly prognostic. Troponin levels, which are reflective of cardiac myocyte death, and NT-proBNP levels, which reflect the degree of ventricular dysfunction, are both routinely measured in patients with patients with newly diagnosed systemic AL

amyloidosis. These cardiac biomarkers in combination with the difference between the involvement and uFLC are used to stratify patients into four prognostic groups in the Revised Mayo 2012 risk score. This difference between the iFLC and uFLC is referred to as the dFLC. Patients with a dFLC <180 mg/L and normal cardiac biomarkers have a median OS of 69 months, however patients with advanced cardiac involvement and a high dFLC at presentation the median OS is only seven months (Kumar *et al*., 2012a). There is also a European modification of the Mayo 2004 risk score which only utilises NT-proBNP and troponin levels (Palladini *et al*., 2015). These risk scores are summarised in Table 1.8.

Table 1. 8 Mayo 2004, revised 2012 Model and the European 2015 modification of the Mayo 2004 risk model for patients with AL amyloidosis

Mayo 2004 Model (Dispenzieri *et al*., 2004)	Criteria	HR for Death
Stage 1	High sensitivity troponin T <50ng/L and NT-proBNP <332 ng/L	reference
Stage 2	Either high sensitivity troponin T ≥50 ng/L or NT-proBNP ≥332 ng/L	2.5 (1.9-3.5)
Stage 3	Both high sensitivity troponin T ≥50ng/L and NT-proBNP ≥332 ng/L	6.7 (5.0-9.0)
Revised Mayo 2012 Model Risk Features		
dFLC ≥180 mg/L		
High sensitivity troponin ≥40 ng/L		
NT-proBNP ≥1800 ng/L		
Revised 2012 Model (Kumar *et al*., 2012a)	**Criteria**	**HR for Death**
Stage 1	No features present	reference
Stage 2	One feature present	1.7 (1.2-2.3)
Stage 3	Two features present	4.1 (3.1-5.5)
Stage 4	All three features present	6.3(4.8-8.3)
European 2015 Modification of the Mayo 2004 Model (Palladini *et al*., 2015)	**Criteria**	**HR for Death**
Stage 1	High sensitivity troponin T <50ng/L and NT-proBNP <332 ng/L	reference
Stage 2	Either high sensitivity troponin T ≥50 ng/L or NT-proBNP ≥332 ng/L	2.5 (1.9-3.5)
Stage 3a	Both high sensitivity troponin T ≥50ng/L and NT-proBNP ≥332-<8500 ng/L	4.9 (3.6-6.8)
Stage 3b	Both high sensitivity troponin T ≥50ng/L and NT-proBNP ≥8500 ng/L	11.1 (8.1-15.4)

The European Association of Haematology and ISA (ISA) guidelines includes broad recommendations about the NT-proBNP and high sensitivity troponin level thresholds that should be used to assess overall patient suitability for ASCT (Sanchorawala *et al*., 2022). The thresholds outlined differ from the thresholds used in the staging systems described above and are used alongside the following additional criteria:

- Age >18 years - <70 years
- Involvement of at least one vital organ (bone marrow and soft tissue involvement are not considered vital organ involvement)
- Left ventricular ejection fraction ≥40% and New York Heart Association functional class <III
- Oxygen saturations ≥95% on room air and gas transfer >50% of predicted on lung function testing
- Supine blood pressure ≥90 mmHg
- Eastern Cooperative Oncology Group performance status ≤2 unless limited only by peripheral neuropathy
- Bilirubin <34 µmol/L
- NT-proBNP <5000 ng/L
- High sensitivity Troponin T <75 ng/ml
- GFR >30 ml/minute/m^2 (patients with end-stage renal disease who are stable on long-term dialysis should not be excluded from ASCT if all of the other eligibility criteria are fulfilled)

In addition, these guidelines incorporate the cardiac staging alongside age and renal function to guide melphalan dosing (Sanchorawala *et al*., 2022). The criteria they recommend are outlined in Table 1.9.

Table 1. 9 EHA and ISA recommendations for ASCT and melphalan dosing in patients with AL Amyloidosis (Sanchorawala *et al*., 2022)

	Melphalan 200mg/m^2 ASCT	MDT discussion re melphalan 200 mg/m2 ASCT versus bortezomib-based regimen	Melphalan 140mg/m^2 ASCT
Age (years)	<65	66-69	-
Cardiac stage	1 or 2	2 or 3	-
eGFR (ml/minute/m^2)	>50	30-50	<30

The BSH guidelines on the diagnosis and investigation of AL amyloidosis also recommend utilising the staging systems based on cardiac biomarkers and FLC levels to identify high-risk patients (Gillmore *et al*., 2015).

Isolated renal involvement is associated with favourable outcomes and patients commonly presents with lower FLC levels (Sidana *et al*., 2019, Dittrich *et al*., 2017). Bortezomib-based treatment regimens are preferred as they have been shown to achieve superior renal outcomes compared to lenalidomide-based regimens (Kastritis *et al*., 2017).

Early and rapid reduction in circulating monoclonal FLC levels is crucial for improving survival and preserving organ function (Palladini *et al*., 2012, Manwani *et al*., 2019, Ravichandran *et al*., 2021, Kastritis *et al*., 2021a). A minimum treatment target is to reduce the dFLC to <40 mg/L (Wechalekar *et al*., 2015) but deeper responses with

reduction of the iFLC to <20 mg/L or reduction of the dFLC to <10 mg/L are associated with the best outcomes (Muchtar *et al*., 2020b). Defining deep responses can be challenging in patients who present with low circulating FLC levels (approximately 20% of patients with amyloidosis have a FLC <50 mg/L at presentation (Milani *et al*., 2017a)) and in patients with renal impairment in whom the polyclonal background light levels will be much higher (Long *et al*., 2022).

The distribution of cytogenetic aberrations observed in patients with AL amyloidosis significantly differs from that seen in patients with multiple myeloma. t(11;14) is the most common cytogenetic abnormality found in AL amyloidosis and is associated with reduced subclone formation (Bochtler *et al*., 2018). t(11;14) has traditionally been associated with poorer outcomes (Bryce *et al*., 2009, Kobayashi *et al*., 2019, He *et al*., 2023), however there is emerging evidence that Daratumumab may be able to be overcome this (Bochtler *et al*., 2016, Kimmich *et al*., 2020). 1q gain and del(17p) both confer an adverse prognosis in patients with AL amyloidosis, however t(4;14) and t(14;16) are infrequently observed (Bochtler *et al*., 2014, Bochtler *et al*., 2016).

1.6 Response Assessment in Patients with Multiple Myeloma

1.6.1 Serological Response Assessments

For the 80-85% of patients with intact-immunoglobulin-secreting disease response is predominantly assessed using SPE and is evaluated at the end of each cycle of induction treatment and every 1-3 months in patients receiving maintenance therapy. The percentage change in the size of the monoclonal protein is used to classify the depth of response using the criteria outlined in the IMWG uniform response criteria

(Durie *et al*., 2006). The IMWG uniform response criteria are described in further detail in Table 2.3.

Owing to the increased sensitivity of IFE, negativity by IFE is required to confirm the absence of any detectable monoclonal protein in patients who have achieved negativity by SPE (Kumar *et al*., 2016). The absence of residual clonal plasma cells on bone marrow biopsy is also required to fulfil the criteria for a complete response (CR) (Kumar *et al*., 2016) and patients who achieve this and have a normal serum FLC ratio are classified as having achieved a stringent CR (sCR).

The serial use of serum FLC assays for monitoring treatment response is currently only recommended for patients with oligo-secretory multiple where there is no other measurable monoclonal protein in the serum. Disease is considered to be measurable by serum FLC assays when the difference between the involved and uFLC is ≥100 mg/L at baseline (Kumar *et al*., 2016). However, in real-world practice serum FLC measurements are commonly used to monitor treatment response in patients with FLC only multiple myeloma even when the level of BJP is measurable at diagnosis as BJP assessments require a 24-hour urine collection, which is logistically complicated and challenging for elderly frail patients. In addition, multiple studies have shown that the serum FLC assays are more sensitive (Drayson M, 2001, McTaggart *et al*., 2013, Dejoie *et al*., 2016).

1.6.2 Limitations of the Current Serological Techniques for Response Assessment

Although the electrophoretic-based assays and serum FLC assessments provide simple, minimally invasive and relatively low-cost methodologies for monitoring response to anti-myeloma therapy, their sensitivity is limited. An sCR represents the deepest response that can be measured using standard response criteria and was added to the IMWG uniform response criteria in 2006 (Durie *et al*., 2006). However, there are conflicting reports on whether normalisation of the serum FLC ratio in patients in CR identifies patients with improved survival (Kapoor *et al*., 2013, Martínez-López *et al*., 2015, Abbi *et al*., 2016, García de Veas Silva *et al*., 2016, Cedena *et al*., 2020, Jiménez-Ubieto *et al*., 2021). These conflicting results may be due to differences in the way patients were classified, including whether the IMWG or EBMT response criteria were applied. With the advances in treatment and subsequent improvement in the quality of responses achieved, it may be that the additional sensitivity provided by serum FLC analysis for low-level residual disease in the serum is less relevant now. In addition, the serum FLC ratio may be affected by non-disease related factors, including suppression of the uFLC by therapy, inflammation, infection and oligoclonal band formation (de Larrea *et al*., 2009, Dispenzieri *et al*., 2009, Heaney *et al*., 2017).

Therapeutic monoclonal antibodies, such as daratumumab, isatuximab and elotuzumab, antibody drug conjugates such as belantamab mafodotin and bispecific T-cell engagers such as teclistamab and elranatmab, are increasingly being used in the treatment of multiple myeloma. These are usually IgG kappa antibodies and can therefore pose difficulties in response assessment for patients with IgG kappa secreting multiple myeloma (Thoren *et al*., 2019), which is the most common

monoclonal protein isotype seen in patients with multiple myeloma (approximately 40% of patients have IgG kappa multiple myeloma).

1.6.3 Minimal Residual Disease (MRD) Testing in Multiple Myeloma

Bone marrow biopsy provides supplementary prognostic information in patients who achieve electrophoretic negativity in the serum and/or urine. Chee *et al* demonstrated that residual clonal plasma cells were detectable in over 10% of patients who had achieved IFE negativity in the serum and urine (Chee *et al*., 2009). However, morphological and immunohistochemical assessments have limited sensitivity and most patients achieving CR eventually relapse. Extensive efforts have therefore been made to develop more sensitive assays, utilising flow cytometry and next generation sequencing (NGS), which can detect low-level residual disease and these bone marrow-based assays were incorporated into the IMWG response criteria in 2016 (Kumar *et al*., 2016).

1.6.3.1 Flow-cytometry based MRD assays using Bone Marrow Samples from Patients with Multiple Myeloma

Flow cytometry based MRD assays identify low-levels of residual disease based on the abnormal phenotype of the neoplastic plasma cell clone. Assay sensitivity is determined by the number of cell events acquired and the number of antibodies used to identify neoplastic plasma cells. Modern assays are able to achieve sensitivities of 1×10^{-5} to 1×10^{-6} (Flores-Montero *et al*., 2017, Roshal *et al*., 2017, Ching *et al*., 2020).

MRD negativity using multicolour flow cytometry has been demonstrated to be associated with improved outcomes in patients with multiple myeloma (Flores-Montero *et al*., 2017, Clark *et al*., 2018, Avet-Loiseau *et al*., 2020, Mohan *et al*., 2021). Studies have demonstrated that the best outcomes are observed in patients achieving the greatest log reduction in MRD (Rawstron *et al*., 2015, Flores-Montero *et al*., 2017, Rossi *et al*., 2019). The IMWG response criteria therefore specify that flow cytometry assays used for MRD assessment should have a minimum sensitivity of 1×10^{-5} (Kumar *et al*., 2016). At this sensitivity threshold, MRD is detectable in 16-52% of patients in CR (Flores-Montero *et al*., 2017, Tschautscher *et al*., 2019, Carulli *et al*., 2019, Schmitz *et al*., 2022, Kim *et al*., 2022, Gozzetti *et al*., 2022).

Serial MRD measurements provide additional prognostically discriminatory information; patients who reach MRD negativity but experience early re-emergence of MRD positivity have reduced progression free survival (PFS) compared to patients with sustained MRD negativity (Munshi *et al*., 2017, Gu *et al*., 2018, Martinez-Lopez *et al*., 2020, Avet-Loiseau *et al*., 2021, Mohan *et al*., 2021, Mohan *et al*., 2022, Fonseca *et al*., 2023, Paiva *et al*., 2023).

Adequate bone marrow aspirate sample quality is crucial to maximise assay sensitivity. Haemodilution is evaluated using the mast cell content of samples when NGF is performed, as haemodilution may lead to false negative MRD results (Flores-Montero *et al*., 2017). Bone marrow aspirate samples should be processed within 24-48 hours of collection as reduced cell viability may also lead to false negative results (Johansson *et al*., 2014).

1.6.3.2 NGS Bone Marrow-Based MRD Assays

NGS assays use amplification and sequencing to determine whether clonotypic immunoglobulin variable region gene rearrangements identified in baseline samples are present at follow-up, confirming the presence of MRD. NGS provides a highly sensitive methodology for MRD detection and has been demonstrated to be able to detect MRD in 39 – 83% of patients on CR (Takamatsu *et al*., 2017, Perrot *et al*., 2018, Avet-Loiseau *et al*., 2021, Cho *et al*., 2022, Ha *et al*., 2022). Multiple studies have demonstrated that NGS MRD-negativity is associated with improved PFS and OS (Martinez-Lopez *et al*., 2014, Takamatsu *et al*., 2017, Perrot *et al*., 2018, Medina *et al*., 2020, Munshi *et al*., 2020, Cho *et al*., 2022), with the best outcomes observed in patients achieving MRD-negativity at a sensitivity threshold of 10^{-6} (Munshi *et al*., 2020).

NGS can be performed both on fresh samples and stored cells and DNA, which provides greater flexibility for collections and sample transit requirements compared to multicolour flow cytometry. However, turnaround times are slower and in 3.3 – 20% of patients, no clonal gene rearrangement is identified at baseline (Ladetto *et al*., 2014, Martinez-Lopez *et al*., 2014, Flores-Montero *et al*., 2017, Yao *et al*., 2020, Cho *et al*., 2022, Hultcrantz *et al*., 2022, Ha *et al*., 2022). In addition, NGS is substantially more expensive than multicolour flow cytometry (NGF costs $800 per sample versus $2100 per sample for NGS analysis using clonoSEQ) (Flores-Montero *et al*., 2017, Walzer *et al*., 2021).

1.6.3.3 Barriers to the Use of Bone Marrow-based MRD Testing in Routine Clinical Practice

MRD assays are widely used in the clinical trial setting and owing to their prognostic utility they are being evaluated as a surrogate marker for survival, which could facilitate earlier regulatory approval of novel therapeutic agents (Munshi *et al*., 2017, Munshi *et al*., 2020). However, their application in real-world practice is variable.

The high-cost of MRD testing limits the frequency of MRD testing and its widespread application. However, Walzer *et al* demonstrated that even MRD testing using the more expensive NGS technique may be cost effective if it can be used to identify patients who may be able to discontinue continuous maintenance therapy (Walzer *et al*., 2021). However, the results of the MASTER trial highlight that other factors in addition to MRD status should be considered prior to discontinuation of therapy as a significantly higher MRD resurgence and/or progression rate was observed in this trial in patients with ≥2 high risk cytogenetic abnormalities (Costa et al., 2021b). At present the use of MRD-guided treatment de-escalation remains an active area of research, which is being explored in multiple ongoing clinical trials (Krishnan *et al*., 2020, Royle *et al*., 2022, Derman *et al*., 2022).

The requirement for bone marrow samples for the currently approved MRD assays also limits testing frequency, as bone marrow sampling is an uncomfortable and invasive procedure. In addition, bone marrow MRD assays sample a single site and may therefore provide false negative results if the residual disease is patchy residual and does not involve the posterior superior iliac spine sampling site. False negative

samples may also be obtained if a poor quality haemodilute aspirate is obtained, which may occur either due to poor positioning of the needle or aspiration of a large volume of marrow. Extensive research has therefore been undertaken to identify more sensitive techniques for response assessment in the peripheral blood as this would provide a more global assessment of the residual disease burden and would provide a minimally invasive and more convenient method for highly sensitive response assessments. The techniques that have been explored to increase the sensitivity for monitoring the disease status of patients with PCD in the peripheral blood are discussed below.

1.7 Novel Methods For Response Assessment in the Peripheral Blood in Patients with Multiple Myeloma

1.7.1 Circulating Tumour DNA (ctDNA)

Liquid biopsies represent an attractive potential methodology for monitoring therapeutic responses in multiple myeloma as they are able to provide a more global assessment of response than single site bone marrow biopsies. In addition, the identification of specific mutations may assist in the personalisation of maintenance strategies and guide subsequent lines of therapy (Manier *et al*., 2018).

Higher amounts of ctDNA at presentation have been shown to be associated with reduced PFS and OS (Ye *et al*., 2022), which may in part be due to the higher levels of ctDNA reported in patients with extramedullary disease (Long *et al*., 2020). However, conflicting results regarding concordance between standard electrophoretic and nephelometric assessments compared to ctDNA response assessment have been

reported (Vrabel *et al*., 2020, Yasui *et al*., 2021, Mack *et al*., 2022, Waldschmidt *et al*., 2022). Concordance may be heavily dependent on the timing of response assessments as the half-life of ctDNA is only a few hours compared to 7-21 days for intact immunoglobulins (Thakral *et al*., 2020).

The potential prognostic utility of monitoring ctDNA has been demonstrated in several studies. Dhakal *et al* reported that patients with no detectable ctDNA post ASCT have improved PFS compared to patients with residual detectable ctDNA (Dhakal *et al*., 2022). Similarly, Waldshmidt *et al* reported that persistently detectable ctDNA after the second cycle of anti-myeloma treatment in patients with relapsed/refractory myeloma was associated with shorter PFS. In this study, ctDNA positivity identified patients with worse PFS who had the same depth of response using the IMWG uniform response criteria (Waldschmidt *et al*., 2022).

Although Vrabel *et al* reported that ctDNA result had better agreement with multicolour flow cytometry-based response assessments than electrophoretic assessments, the sensitivity of ctDNA compared to multicolour flow cytometry in bone marrow was only 67% (Vrabel *et al*., 2020). Mazzotti *et al* also reported similar results with no residual disease being detectable by ctDNA in 69% of patients with detectable MRD in the bone marrow by NGS (Mazzotti *et al*., 2018). The bone marrow assays therefore provide greater sensitivity than the currently available ctDNA assays. However, liquid biopsies may provide a useful tool for monitoring patients with extramedullary disease (Mithraprabhu *et al*., 2017) and/or low-level level secretory disease and may provide a useful guide for the timing of the bone marrow and imaging assessments.

1.7.2 Peripheral blood flow cytometry

Plasma cells are highly dependent on stromal cell and cytokine support from the bone marrow microenvironment, so the peripheral blood provides a harsh environment for their proliferation. However, cells with downregulation of cell adhesion molecules and integrins may survive. Consequently, low numbers of circulating plasma cells are found in the peripheral blood of the majority of patients with active multiple myeloma (Sanoja-Flores *et al*., 2018, Wang *et al*., 2019).

Dingli *et al* demonstrated that the presence of circulating neoplastic plasma cells detectable by multicolour flow cytometry was associated with reduced time to progression and OS. In this study, the CR rates did not significantly differ between the two patient cohorts and patients in CR without MRD negativity had similar outcomes to patients in PR (Dingli *et al*., 2006)

Although CD38/CD138 enrichment has been demonstrated to improve assay sensitivity, Wang *et al* still found that 12% of patients with detectable MRD in the bone marrow by flow cytometry (assay sensitivity 10^{-4}) had no MRD detectable in the peripheral blood by flow cytometry (Wang *et al*., 2019).

1.7.3 Hevylite

Hevylite is a nephelometry/turbidimetry-based serum assay which separately quantitates the total amount of each heavy chain class bound to kappa and lambda light chains. It has been demonstrated to provide good linearity for the quantitation of low-level monoclonal proteins (<10g/L at presentation) and the accuracy of quantitative

results is not affected in patients with beta-migrating IgA monoclonal proteins (Ludwig *et al*., 2013, Boyle *et al*., 2014).

Hevylite has high diagnostic sensitivity for the detection of multiple myeloma at presentation (Ríos-Tamayo *et al*., 2021) and overall good concordance with electrophoretic techniques for response assessment has been reported (D'Souza *et al*., 2017). However, greater discrepancies have been observed amongst patients classified as ≥VGPR using standard electrophoretic techniques with conflicting results regarding its prognostic significance . Michallet *et al* reported that the persistence of abnormal HLC ratio was associated with reduced PFS in patients in >VGPR and better concordance with low-sensitivity (1×10^{-4}) flow cytometry-based bone marrow response assessments (Michallet *et al*., 2018). However, Ludwig *et al* and D'Souza *et al* reported no significant improvement in outcomes for patients achieving normalization of their Hevylite ratio who were classified as being in >VGPR using standard electrophoretic techniques (Ludwig *et al*., 2013, D'Souza *et al*., 2017).

The use of Hevylite testing in routine clinical practice remains limited due to the higher reagent costs and limited testing availability. However, it may have utility in patients with oligo-secretory intact immunoglobulin secreting multiple myeloma due to its lower reported limit of detection compared to SPE (Ríos-Tamayo *et al*., 2021).

1.7.4 Modified IFE

The electrophoretic assays currently employed in routine clinical practice have limited sensitivity for the detection of FLC monoclonal proteins due to the lack of FLC specific

reagents. Singh *et al* demonstrated that utilising ultrafiltration with a size exclusion threshold of 50 kDa to remove intact whole immunoglobulins molecules prior to IFE analysis can enhance the limit of detection of IFE down to 0.7mg/L and 4 mg/L for monoclonal lambda and kappa FLC respectively, even in the absence of FLC specific reagents (Singh and Bollag, 2020). Wilhite *et al* demonstrated similar sensitivity utilising antisera specific for kappa and lambda FLC in combination with IFE (Wilhite *et al*., 2021). Both of these studies only included small numbers of patients samples, so analysis of the prognostic implications of residual positivity by these techniques was not reported and at present the modified IFE assays remain a research tool.

1.7.5 Serum B-cell Maturation Antigen (BCMA)

BCMA is a pro-survival antigen, which is much more highly expressed on neoplastic plasma cells compared to healthy plasma cells and is the antigenic target for multiple novel agents in multiple myeloma. Serum BCMA increase as MGUS progresses to multiple myeloma and levels has been shown to correlate with disease status and survival (Sanchez *et al*., 2012). Ghermezi *et al* reported good correlation between plasma cell infiltration in the bone marrow and serum BCMA levels in patients with multiple myeloma and good correlation with PET-CT findings in patients with non-secretory multiple myeloma (NSMM) (Ghermezi *et al*., 2017). It may potentially provide a useful future serum biomarker for the serological monitoring patients who cannot be monitored using electrophoretic techniques. To date there are no published studies demonstrating that monitoring serum BCMA levels provides superior prognostic information to the currently utilised electrophoretic assays and therefore it remains a research tool at present.

1.7.6 Imaging-based Response Assessments in Multiple Myeloma

Spatial heterogeneity is a common feature in multiple myeloma and subclones at different sites may have distinct genomic profiles, leading to differential treatment sensitivity (Mithraprabhu *et al*., 2017, Rasche *et al*., 2019, Garcés *et al*., 2020). In addition, 2.4-20% of patients have extramedullary disease at presentation (Bladé *et al*., 1996, Usmani *et al*., 2012, Zamagni *et al*., 2015, Bailly *et al*., 2018, Li *et al*., 2019, Danhof *et al*., 2021) and an increasing proportion of patients develop extramedullary disease at relapse (Usmani *et al*., 2012). Extramedullary disease is a high-risk feature, which is associated with high-risk cytogenetic abnormalities and reduced survival (Varettoni *et al*., 2010, Usmani *et al*., 2012, Avivi *et al*., 2019, Han *et al*., 2021). There has therefore been great interest in whether imaging-guided response assessments can provide additional prognostic information.

MRI provides the greatest sensitivity for the detection of bone marrow lesions (Derlin *et al*., 2013, Regelink *et al*., 2013, Messiou *et al*., 2021, Westerland *et al*., 2021a) and is recommended as second-line imaging in patients with a negative low-dose whole body CT (Hillengass *et al*., 2019). However, there is a need to avoid repeated imaging at diagnosis and ideally a whole body diffusion weighted MRI (DW-MRI) is sufficient as a single imaging test and should be the first-line imaging test for patients with multiple myeloma. However, the implementation of MRI as a means for assessing therapeutic responses and assist in residual detection has been limited by high false positive rates (Derlin *et al*., 2013, Basha *et al*., 2018). Recent studies have highlighted that this may have been due the frequent absence of diffusion-weighted imaging and have demonstrated that the inclusion of these imaging sequences can aid in the differentiation between active and inactive lesions (Messiou *et al*., 2021).

In a study by Rasche *et al*, residual focal intensities on DW-MRI in patients in CR was associated with reduced PFS, particularly in patients with high-risk cytogenetic abnormalities (Rasche *et al*., 2019). This study also demonstrated that the combination of bone marrow MRD and functional imaging improved the detection of residual disease; 4/83 patients were MRD negative but had residual focal lesions and two of these patients experienced early relapse (Rasche *et al*., 2019). However, its widespread implementation may be restricted by capacity issues (Westerland *et al*., 2021b).

Although 18-FDG PET-CT is less sensitive than MRI for the detection of intramedullary disease (Messiou *et al*., 2021, Kaiser *et al*., 2021), it has been demonstrated to have higher specificity for residual disease than MRI (Derlin *et al*., 2013, Basha *et al*., 2018). However, false positives may still occur due to increased uptake due to other pathologies such as microfractures.

PET-CT also provides additional prognostic information for patients who have achieved bone marrow MRD negativity, with the longest PFS being observed in patients who are MRD negative in the bone marrow and on functional imaging (Moreau *et al*., 2017, Rasche *et al*., 2019, Alonso *et al*., 2019). Due to these encouraging results PET-CT was incorporated into the 2016 updated IMWG for response and MRD assessment in multiple myeloma (Kumar *et al*., 2016).

1.7.7 Mass Spectrometry (MS) Assays for the Measurement of Monoclonal Proteins in Peripheral Blood

MS-based assays have emerged in the last decade as a potentially novel method for monitoring therapeutic responses in the peripheral blood of patients with PCD (Moore *et al*., 2019, Eveillard *et al*., 2021, Noori *et al*., 2021, Kohlhagen *et al*., 2021, Santockyte *et al*., 2021). MS is an analytical technique in which molecules are converted to gas phase ions and are separated according to their mass-to-charge (*m/z*) ratio and the results are displayed on mass spectra. All types of mass spectrometer consist of three basic components. The *ion source*, converts the sample molecules into ions; *mass analysers* separate the ions according to their *m/z*; and the *detector* measures the abundance of ions (Greaves and Roboz, 2013).

Three different mass spectrometry approaches have been explored as novel ways for the detection and monitoring of monoclonal proteins in patients with PCD. In the first two approaches, the assays track the light chain portion of the monoclonal protein as an intact protein and are therefore referred to as intact light chain assays. Having undergone light chain VJ gene rearrangement and somatic hypermutation the light chain produced by the clonal plasma cell population will have a unique amino acid sequence and therefore a specific *m/z* and light chain isotype which can be used to monitor the monoclonal protein throughout the course of anti-myeloma treatment.

The light chain portion of the monoclonal protein has been selected for tracking the monoclonal protein in the MS assays as in contrast to the heavy chain they are not routinely glycosylated and they are smaller meaning they will ionise more readily (Mills

et al., 2015). In addition, the light chain mass range is consistent across all of the monoclonal protein isotypes whereas the intact immunoglobulin molecules have significantly different masses, which would require different assay conditions. For example, a matrix-assisted laser desorption/ionisation time-of-flight MS (MALDI-TOF MS) assay analysing intact IgG molecules as opposed to light chains would require data to be collected over a different *m/z range,* a different protein calibration mix appropriate for the *m/*z range being used, different laser power and an alternative matrix that was suitable for larger proteins (Frecklington, 2007).

In the intact light chain assays the samples are enriched for immunoglobulins, which enhances assay sensitivity as it reduces the background noise over which any monoclonal peaks will be observed. This may be achieved using gel separation (Ladwig *et al*., 2017) or immunoprecipitation using either camelid-derived nanoparticles (Murray *et al*., 2019) or magnetic microparticles (Sakrikar *et al*., 2021) coated with antisera for the constant regions of the relevant immunoglobulin heavy and/or light chain isotypes. The purified immunoglobulins are reduced to release the bound light chains from the heavy chain in intact immunoglobulin molecules and the resulting eluates are then analysed by MS.

The intact light chain approach has been applied using two different forms of MS – MALDI-TOF MS and liquid chromatography MS (LC-MS) . The MALDI-TOF MS assays provide a higher throughput MS methodology, which provides greater sensitivity than the currently widely utilised electrophoretic techniques, where workflows that permit automated sample processing and analysis of spectra are being

developed. The MALDI-TOF MS-based intact light chain assays may therefore represent the most practical option as a potential replacement for the currently utilised electrophoretic assays for routine clinical use (Kohlhagen *et al*., 2020, Santockyte *et al*., 2021).

In MALDI-TOF MS, samples are combined with a matrix and spotted onto target plates. As the matrix dries the light chains are embedded within it and co-crystallized. When the sample is analysed each dried spot is exposed to a sudden burst of excess energy as the laser hits the sample spot and the matrix then transfers a charged species, usually a proton, to the light chain resulting in the formation of charged light chain ions. The light chain ions formed are most commonly singly and doubly charged light chain ions. The ions are accelerated from the source and the time taken for the ion to reach the detector is measured and ions with the lowest *m/z* will reach the detector first. The results are displayed in a mass spectrum in which the peaks are separated on the x-axis according to their *m/z.* The intensity of each peak, which represents the relative abundance of each molecule, is displayed on the y-axis (Greaves and Roboz, 2013). An example mass spectrum is shown in Figure 1.5.

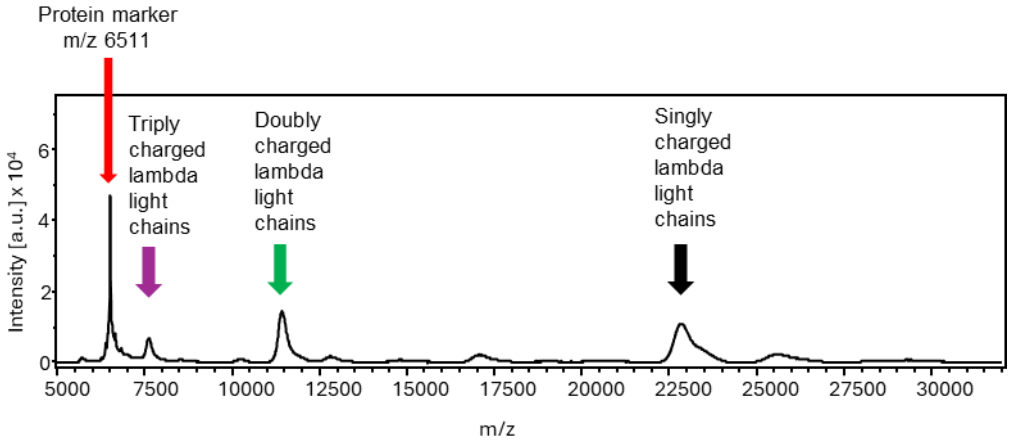


Figure 1. 5 Example mass spectrum from a polyclonal sample that has undergone immune enrichment with total lambda light beads prior to analysis by MALDI-TOF MS

The m/z is displayed along the x-axis and the intensity is displayed along the y-axis. The peaks representing the singly, doubly and triply charged polyclonal lambda light chains in this sample are highlighted by the black, green and purple arrows respectively. The peak with the highest intensity in this mass spectrum is the peak at m/z 6511 (highlighted by the red arrow), which is due to the protein marker which is used as a control check for the spotting step of the assay.

In the intact light chain LC-MS based assays, the eluates obtained after immune enrichment undergo separation using liquid chromatography prior to ionisation. This results in much longer analytical times as the chromatographic separation takes 10-25 minutes per sample (Mills *et al*., 2015, Santockyte *et al*., 2021, Giles *et al*., 2022). Ionisation is performed by electrospray ionisation (ESI), which is an alternative form of soft ionisation to MALDI in which an electrical current is used to generate gaseous phase ions from a solution. ESI generates much higher rates of multiple charged ions than MALDI, so specialised software is needed to deconvolute the spectra and identify the neutral mass of the intact protein. At present all total ion chromatograms and deconvoluted are manually analysed and therefore the throughput of these assays is significantly lower than the throughput of the MALDI-TOF MS assays.

The Mayo clinic were the initial pioneers of the intact light chain technique and have explored its utility on both MS platforms. The MALDI-TOF MS based intact light chain assay developed by the Mayo clinic is known as the MASS-FIX assay. The MASS-FIX has replaced the use of IFE at the Mayo clinic following guidance from the IMWG that stated that intact light chain assays could be used in lieu of IFE (Murray *et al*., 2021). The initial analysis and clinical roll-out of this assay only utilised it for qualitative analysis (Kohlhagen *et al*., 2020). However, in early 2024 the Mayo clinic also started performing quantitative analysis with MASS-FIX, with a limit of quantitation of 100mg/L (Mayocliniclabs, 2024), on routine clinical samples. This assay has only been developed to a laboratory diagnostic test specification and therefore does not have regulatory approval to be sold as kits and equipment for use at centres across the globe. The Mayo Clinic have also developed an LC-MS based intact light chain assay, which has a limit of detection of 5 mg/L, which is known as the miRAMM assay (Mills *et al*., 2015).

The first commercially available MS-based assay is the EXENT assay (formerly referred to as QIP-MS), which has been developed by The Binding Site, UK, part of Thermo Fisher Scientific. In contrast to the MASS-FIX assay, which uses camelid-derived nanoparticles and gravitational separation during the immune enrichment stage, the EXENT assays uses paramagnetic particles and magnetic plates. MASS-FIX and EXENT utilise different acquisition settings and distinct in-house developed software for the analysis for the analysis of the spectra generated. There are no published studies of the relative sensitivity of these two assays but reported lower limit of measurement for the EXENT assay is lower (15mg/L for intact immunoglobulin

monoclonal proteins) (Sakrikar *et al*., 2021). Both the standard EXENT assay and the MASS-FIX assay use a five bead panel, which has beads specific for IgG, IgA, IgM, total kappa and total lambda. The workflow for the intact light chain assays is summarised in Figure 1.6A.

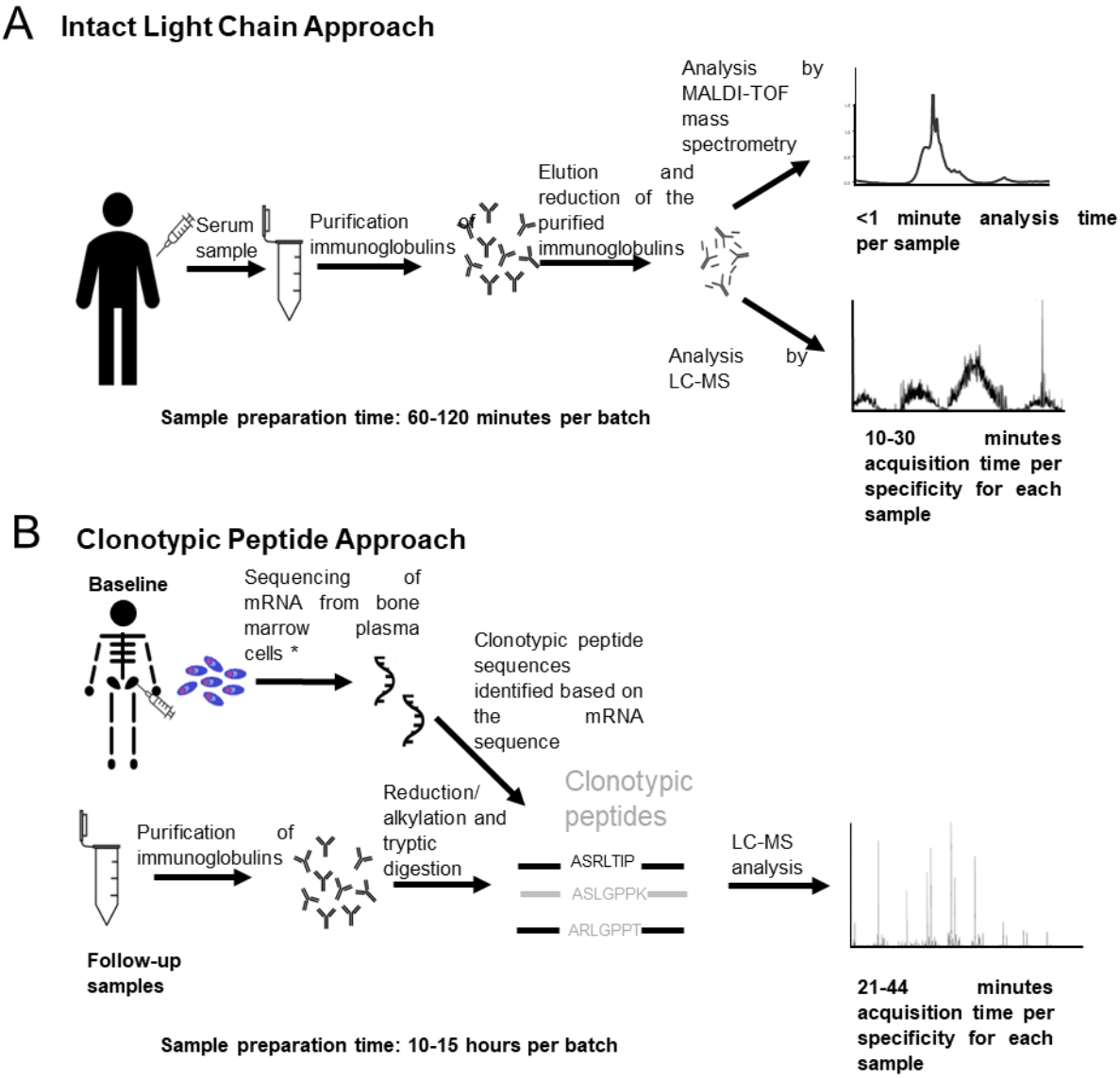


Figure 1. 6 Experimental workflow for the intact light chain and clonotypic mass spectrometry assays

The workflow for the intact light chain assays is shown in (A). Samples can be prepared manually or in batches of 16-64 samples for the five bead assays. The batch size depends on the assays and the model of liquid handler used (Kohlhagen et al., 2020, Barnidge et al., 2024). Eluted and reduced samples are spotted onto target plates for MALDI-TOF MS analysis or transferred to glass vials for LC-MS analysis. The workflow for the clonotypic peptide assays is shown in (B).

**Many authors have used have used mRNA sequencing from bone marrow plasma cells to identify suitable clonotypic peptide sequences but this step is not required for the EasyM assay provided the monoclonal protein is ≥2g/L at presentation (Liyasova et al., 2021).*

Figure reproduced from: Giles et al. The potential role of mass spectrometry for the identification and monitoring of patients with plasma cell disorders: where are we now and which questions remain unanswered? British Journal of Haematology 2022; 198(4): 641-653.

A companion assay to EXENT, which uses antisera specific for free kappa and free lambda light chains, has also been developed and is commonly referred to as FLC-MS. The antisera used in this assay targets a portion of the FLC constant region which is hidden when light chain are bound to heavy chains as part of whole immunoglobulin molecules (Figure 1.7).

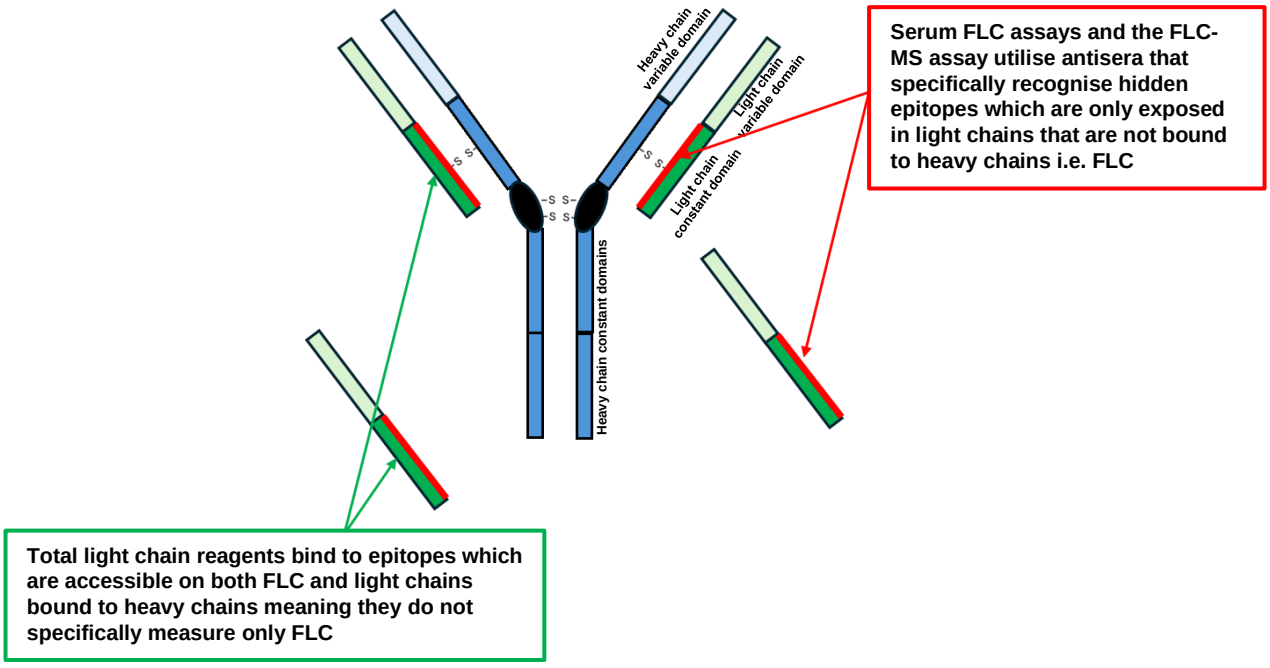


Figure 1. 7 The difference between total light chain and FLC specific reagents
FLC specific reagents bind to a region of the constant domain of light chains that is only accessible for reagent binding when the light chain is not bound to a heavy chain (highlighted by the red arrows). In contrast total light chain assays bind to the light chain constant region in a site that is accessible both when the light chains are free and when they are bound to heavy chains as part of an intact immunoglobulin molecule (highlighted by the green arrows).

Figure adapted from Giles HV and Karunanithi K. Performance Characteristics and Limitations of the Available Assays for the Detection and Quantitation of Monoclonal Free Light Chains and New Emerging Methodologies. Antibodies 2024; 13(1):19.

The final MS-based technique that has been explored for the identification and monitoring of monoclonal proteins in patients with PCD are the clonotypic assays. These assays use specific peptide sequences identified either by comparison with

sequencing data from a baseline bone marrow sample (Barnidge *et al*., 2014) or the monoclonal protein if it is present at a sufficiently high concentration in the baseline sample (Liyasova *et al*., 2021) as the biomarker that is tracked by the MS assay. The workflow for the clonotypic assays is summarised in Figure 1.6B. Absolute quantitation of the monoclonal protein can be obtained by using stable isotope labelled peptides (Zajec *et al*., 2020). The clonotypic peptide method has the lowest limit of detection reported - 0.5-1.5 mg/L (Zajec *et al*., 2018, Martins *et al*., 2020, Liyasova *et al*., 2021, Langerhorst *et al*., 2021).

The prognostic utility of the clonotypic peptide approach has yet to be demonstrated as the studies exploring its potential utility have only included small numbers of patient samples. Its suitability for widespread implementation as a routine methodology for MRD assessment remains uncertain as there are high upfront costs for the equipment required to perform the analysis. In addition, the long pre-analytical and analysis times for each sample may mean it is impractical for use in high throughput clinical laboratories.

Table 1.10 summarises the MS assays that have been developed for the detection and monitoring of monoclonal proteins in patients with PCD.

Table 1. 10 Summary of the MS-based assays developed to detect and monitor monoclonal proteins

Assay Name	Enrichment method	Specificity of Antisera	Analyte	Type of MS Analysis	Sensitivity	Cost per sample
MASS-FIX (Mills *et al*., 2016, Mayocliniclabs, 2024)	Immune enrichment using camelid nanoparticles	IgG, IgA, IgM, total kappa and total lambda	Intact light chain	MALDI-TOF-MS	LOD <100 mg/L LOQ 100 mg/L	$145
QIP-MS/EXENT (Sakrikar *et al*., 2021)	Immune enrichment using magnetic microparticles	IgG, IgA, IgM, total kappa and total lambda	Intact light chain	MALDI-TOF MS	LOQ 15 mg/L	$200-250
FLC-MS (Sharpley *et al*., 2019a)	Immune enrichment using magnetic microparticles	Free kappa and free lambda	Intact light chain	MALDI-TOF MS	Not published	Not published
MiRAMM (Mills *et al*., 2015)	Melon gel	IgG, IgA, IgM, total kappa and total lambda	Intact light chain	LC-MS	LOD 5 mg/L	Not published
EXENT LC-MS (Derman *et al*., 2021, Barnidge *et al*., 2024)	Immune enrichment	IgG, IgA, IgM, total kappa and total lambda	Intact light chain	LC-MS	LOD 1 mg/L	Not published
EXENT FLC LC-MS (Puig N, 2021)	Immune enrichment	Free kappa and free lambda	Intact light chain	LC-MS	Not published	Not published
EasyM (Fan *et al*., 2024)	Precipitated out with ammonium sulfate with concentration gradient	N/A	Clonotypic peptide	LC-MS	LoQ 0.6-2.0mg/L	Not published
Non-branded kappa and lambda enrichment clonotypic peptide assay (Martins *et al*., 2020)	Immune enrichment using Capture Select Kappa-XL Affinity Matrix or Lambda resins in a spin filter	Kappa and Lambda	Clonotypic peptide	LC-MS	0.1-1.5 mg/L	Not published

1.8 Response Assessment in AL Amyloidosis

Response to treatment in patients with AL amyloidosis is categorised according to haematological criteria and organ response criteria. The current haematological response criteria are predominantly defined based on the degree of reduction of the monoclonal FLC. For an amyloid CR the FLC must have normalised and there must be no residual monoclonal protein detectable by IFE in the serum or urine (Palladini *et al*., 2012). Patients in whom the FLC ratio is abnormal post-treatment due to the uFLC level being higher than the amyloidogenic FLC should also be classified as being in CR if they fulfil the other criteria for CR as this phenomenon is likely due to treatment-related immune suppression (Palladini *et al*., 2021b). The full details of the haematological response criteria currently in use are listed in Table 2.6.

Organ responses are defined according to improvements in biomarkers of organ function in the absence of deterioration in organ function. NT-proBNP is used for cardiac response; proteinuria is used for renal response; and alkaline phosphatase levels are used for hepatic response. The details of the organ response criteria currently in use are listed in Table 2.5.

Sidana *et al* have recently validated a composite haematological and organ response (CHOR) model, which combines the depth of haematological response and proportion of organs which have shown response to therapy. The CHOR model had superior predictive power for OS compared to when haematological or organ response were used in isolation and low CHOR scores at 6 and 12 months post treatment initiation were associated with improved OS (Sidana *et al*., 2020b).

There are ongoing efforts to define further subcategories of organ response as data are emerging demonstrating that greater depths of organ response are associated with improved outcomes (Muchtar *et al*., 2018).

1.9 Novel Methods for Response Assessment in Amyloidosis

1.9.1 Multiparametric Flow Cytometry Bone Marrow-based MRD Assessments

MRD testing on bone marrow samples using multiparameter flow cytometry has been shown to be a highly sensitive methodology for MRD detection and can detect residual disease in 17.6-60% of patients in haematological CR (Kastritis *et al*., 2018, Sidana *et al*., 2020c, Staron *et al*., 2020, Palladini *et al*., 2021a, Li *et al*., 2021). MRD positivity amongst patients who have achieved deep haematological responses has been shown to be associated with reduced PFS (Sidana *et al*., 2020c, Muchtar *et al*., 2020a, Palladini *et al*., 2021a, Li *et al*., 2021).

The results regarding the relationship between MRD negativity and organ response have been more variable. Utilising multiparametric flow cytometry assays with a minimum sensitivity of 1×10^{-5} , Palladini *et al* and Sidana *et al* found a higher rate of cardiac response amongst MRD negative patients (Sidana *et al*., 2020c, Palladini *et al*., 2021a). Li *et al* also found that MRD negativity with a sensitivity of 5×10^{-5} was also associated with increase cardiac response rates. Trends towards improved cardiac response patients who reached MRD negativity were also reported by

Chakraborty *et al* and Muchtar *et al* (Muchtar *et al*., 2020a, Chakraborty *et al*., 2022). However, Staron *et al* reported no significant difference in the rate of cardiac response between MRD negative and positive patients. (Staron *et al*., 2020).

The study by Palladini *et al* is the only study to have reported a significant difference in the rate of renal response rate between patients achieving MRD negativity versus MRD positive patients at a minimum sensitivity of 1×10^{-5} (Palladini *et al*., 2021a). Staron *et al* also reported a trend towards improved renal for patients achieving MRD negativity at 1×10^{-5} (Staron *et al*., 2020), however Chakraborty *et al* reported that MRD status had no significant impact on renal response rates (Chakraborty *et al*., 2022).

1.9.2 NGS Bone Marrow-based MRD Assessments

NGS MRD testing of bone marrow samples has been less extensively studied in AL amyloidosis compared to multiparametric flow cytometry. In a small study of 36 patients, Sarosiek *et al*. found trackable sequences in 86% of patients in the bone marrow using NGS at baseline. In addition, 92% of patients in ≥VGPR with an available follow-up sample had detectable MRD by NGS. However, the rate of clonal sequence detection in the peripheral blood in this study was low – only 11% of patients had a trackable sequence identified in their baseline sample (Sarosiek *et al*., 2021). This may be due to the much low plasma cell burden observed in patients AL amyloidosis compared to those with multiple myeloma.

1.9.3 Functional Cardiac Response Assessments

Cardiac involvement in systemic AL amyloidosis is a significant determinant of prognosis and a major cause of early mortality. Cardiac involvement and response are assessed using cardiac biomarkers (Troponin and NT-proBNP) and echocardiographic assessments. However, echocardiographic changes lag behind cardiac biomarkers (Madan *et al*., 2012) and are therefore of limited use when assessing if patients require early switch of treatment. Multiple novel functional tests have therefore been explored in an attempt to identify tests of cardiac function that display earlier response kinetics compared to the standard echocardiographic criteria.

The six-minute walk distance test is a simple functional test that measures the distance a patient can walk on a hard flat surface within six minutes. It has been shown to be of prognostic value at presentation in patients with AL amyloidosis (Pulido *et al*., 2017, Flatman *et al*., 2017, Cohen *et al*., 2022) and improvements in six minute walking distance have been shown to correlate well with cardiac biomarker response (Decker *et al*., 2017). Cohen *et al* also demonstrated that improvements in six minute walking distance were associated with improved survival (Cohen *et al*., 2022) but this test has not yet been incorporated into cardiac response criteria.

Longitudinal strain represents the shortening in the distance between the left ventricular base and apex which occurs with every heartbeat. Salinaro *et al* demonstrated that normalisation of the apical to basal strain ratio occurred in the absence of significant changes in wall thickness and ejection fraction (Salinaro *et al*., 2017) and is currently being explored as a marker of cardiac response.

1.9.4 MS-based Response Assessments in AL Amyloidosis

At the time of submission of this PhD thesis only one small study of 33 patients by Dispenzieri *et al* had been published as a full paper exploring the prognostic implications of MS-based assessments. In this study, residual monoclonal FLC were detectable by MS in 12% of patients in an amyloid CR and MS positivity was associated with reduced time to progression. However, in half of the MS positive patients residual monoclonal FLC were only detectable by LC-MS (Dispenzieri *et al*., 2020a). There were no published papers exploring the relationship between organ response and MS-based response assessment in patients with AL amyloidosis.

1.10 Protein Glycosylation

1.10.1 Overview of Protein Glycosylation

Protein glycosylation is a highly significant and common form of post-translational modification, which is estimated to be present in over 50% of proteins in biological systems (Apweiler *et al*., 1999). It is an enzymatically controlled process (Chang *et al*., 2011) that plays a critical role in protein structure, stability, solubility and function (Reily *et al*., 2019).

There are several different types of protein glycosylation but N-linked and O-linked are the most prevalent. In N-linked glycosylation the glycosylation site is the amide nitrogen atom of asparagine and the core is always composed of two N-acetylglucosamine residues followed by three mannose residues (Figure 1.8). Variable numbers of other monosaccharides (typically 7-25 residues) are covalently bonded to this core to form highly complex and branched glycoproteins. In O-linked glycosylation,

the glycans are attached to the oxygen atom on the hydroxyl group of either threonine or serine. In general, N-glycans are longer and more complex structures than O-glycans. (Reily *et al*., 2019).

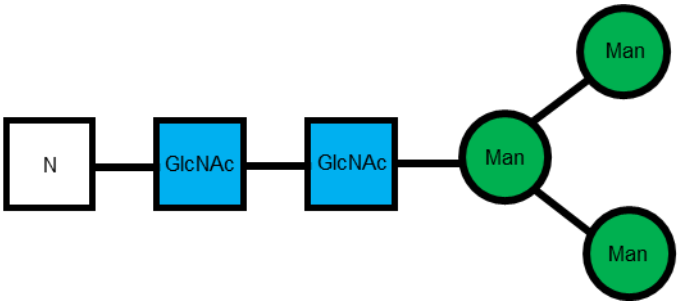


Figure 1.8 The common pentasaccharide core of all N-linked glycans
N-glycans all contain a pentasaccharide core with an N-acetylglucosamine (GlcNAc) attached to the nitrogen atom of the amino group of an asparaginine (N) by a β1-glycosidic linkage. The core consists of two GlcNAc residues and three mannose (Man) residues.
Figure adapted from: Reilly et al. Glycosylation in health and disease. Nature Reviews Nephrology 2019; 15; 346-366.

1.10.2 Process of N-linked Glycosylation and Types of N-linked Glycans

N-glycan synthesis takes place in two stages, the first of which involves a highly conserved pathway (Reily *et al*., 2019). In the first stage, a large oligosaccharide is transferred to an asparagine residue in the endoplasmic reticulum (Kiely *et al*., 1976). The second stage starts in the lumen of the endoplasmic reticulum and continues in the Golgi apparatus. This stage involves glycosyltransferases and glycosidases, which are differentially expressed in different cell types and their activity is determined by the conditions within the cell (Roth, 1987, Reily *et al*., 2019).

The resulting N-linked glycans can be divided into three subgroups. Firstly, *high-mannose N*-glycans which only contain mannosyl residues in addition to the pentasaccharide core. Secondly, *complex type N*-glycans which contain variably

extended antennae, resulting in bi- tri and tetra-antennary structures. Lastly, there are *hybrid* type *N*-glycans whose structure and complexity share features of both *high-mannose* and *complex type N*-glycans (Esmail and Manolson, 2021).

1.10.3 Significance of *N*-linked Light Chain Glycosylation in Patients with PCD

Immunoglobulin molecules are variably glycosylated glycoproteins (Taylor and Wall, 1988, Lee *et al*., 1990, Leibiger *et al*., 1998, Leibiger *et al*., 1999). IgG has a conserved glycosylation site at Asp297 in the CH2 region whereas IgA, IgM, IgD and IgE have multiple conserved glycosylation sites (Reily *et al*., 2019). In contrast there are no conserved glycosylation sites within the constant regions of kappa or lambda light chains (Spiegelberg *et al*., 1970). Variable domain light chain glycosylation sites are found in approximately 5% of light chains in polyclonal immunoglobulins but are found at an increased frequency in patients with PCD (Langerhorst *et al*., 2024), particularly patients with AL amyloidosis (Kumar *et al*., 2019, Dispenzieri *et al*., 2020b, Mellors *et al*., 2021, Dasari *et al*., 2021, Nevone *et al*., 2022, Langerhorst *et al*., 2024). Altered spatial and sequence patterns of N-linked glycosylation on patients with kappa light chain AL amyloidosis have been described, highlighting that N-linked glycosylation may play a potential role in the pathogenicity of some amyloidogenic FLC (Stevens, 2000, Nevone *et al*., 2022).

Light chain N-linked glycosylation is easily identifiable using the intact light chain MS-based assays and appears as polytypic peaks with an increased *m/*z compared to non-glycosylated polyclonal and/or monoclonal proteins (an example mass spectrum of a monoclonal light with N-linked glycosylation in shown in Figure 2.4). In addition to

being found at increased frequency in patients with AL amyloidosis, light chain N-linked glycosylation has been shown to be associated with an increased risk of progression from MGUS to symptomatic PCD, independent of the Mayo MGUS risk score (Dispenzieri *et al*., 2020b). However, no studies have evaluated the impact of light chain N-linked glycosylation on presenting features and patient outcomes in patients undergoing treatment for symptomatic PCD.

1.11 Aims of this study

The aims of this PhD thesis are threefold: firstly, to contribute towards the development of an automated MALDI-TOF MS based assay for the detection and monitoring of patients with PCD and identify the optimal testing panel for the clinical studies undertaken as part of this PhD thesis; secondly, to explore the clinical utility of MS-based assays in patients with multiple myeloma and AL amyloidosis; and thirdly, to evaluate the prognostic significance of light chain N-linked glycosylation in patients with AL amyloidosis and multiple myeloma;. Patients with lymphoplasmacytic lymphoma were not evaluated as this is a lymphoproliferative disorder and the focus of the studies included in this thesis was patients with patients with PCD.

The specific objectives of this PhD thesis, which will enable this overall aims to be addressed are as follows:

- To have a major role in the laboratory development of an automated MALDI-TOF MS based assay for the detection and measurement of monoclonal proteins in patients with suspected PCD to a standard where it will be launched commercially. This will facilitate the identification of the most suitable sample

type/s for MS testing in the clinical studies and also the most suitable reagent panel to use for the testing.

- To evaluate the sensitivity and prognostic utility of MALDI-TOF MS for response evaluation in patients with newly diagnosed multiple myeloma compared to the standard electrophoretic techniques and bone marrow-based MRD assessments. This objective will be addressed using aliquots of stored samples from patients treated in the Myeloma XI trial. Stored rather than freshly collected samples were chosen as due long duration of median PFS achieved with modern regimens, it would not be possible to evaluate the prognostic utility of these assays using samples collected from a single NHS institution of the course of a 3 year PhD project. The timepoints for the samples tested in this project were chosen to match the timepoints at which central laboratory assessments, including bone marrow MRD assessments were conducted as part the Myeloma XI trial. An additional later time point of 15 months post ASCT at which time there were no paired MRD assessments were performed was also chosen to evaluate the impact of the long half-life IgG on the timing of maximum response.

- To evaluate the potential utility of MS-based assays for the detection and monitoring of patients with newly diagnosed NSMM and oligo-secretory myeloma using samples from patients treated in the Myeloma XI trial. Additional baseline samples from patients with serologically non-measurable PCD diagnosed at UHB in whom histology results were available were also

prospectively collected and analysed to explore whether the low-level monoclonal proteins identified corresponding to the immunoglobulin expression patterns identified on immunohistochemistry.

- To compare the sensitivity of MALDI-TOF MS to the standard electrophoretic techniques and serum FLC assessment in patients undergoing treatment for systemic AL amyloidosis and to evaluate the prognostic implications and potential for monitoring patients using MS-based response assessments. Samples from baseline were tested to establish sensitivity of the assay at baseline and also to identify the isotype and *m/z* of the amyloidogenic FLC. Follow-up samples from six months and twelve months when SPE, IFE and Freelite® and organ biomarker results were available were evaluated to assess the sensitivity and prognostic utility of FLC-MS based response assessments in patients with AL amyloidosis. These results were also used to assess the impact of FLC-MS response on organ responses.

- To evaluate the prognostic significance of light chain N-linked glycosylation in patients undergoing treatment for symptomatic newly diagnosed multiple myeloma and AL amyloidosis using the baseline samples tested from patients in the Myeloma XI trial and the ALchemy study.

2: MATERIALS AND METHODS

2.1.Declaration

This thesis contains four clinical studies exploring the potential utility of MS-based assays for the detection and monitoring of patients with PCD. During this period I was heavily involved in the laboratory development and optimisation of MS-based assays. I designed all of the studies that utilised samples from the Myeloma XI trial and I prepared a written proposal for these studies that was reviewed and approved by the UK Myeloma Trials Management Group. I set up the Material Transfer Agreement (MTA) for these samples to be transferred to The Binding Site Ltd, which is part of Thermo Fisher Scientific, in Edgbaston.

The sample identification numbers for the baseline, post cycle 1 (when no baseline was available), end of induction and day+100 post ASCT were identified directly from the Myeloma XI central laboratory database. The samples from the six month post maintenance timepoint and 15 months post ASCT were identified from the master list of the 125000 samples that have been received for patients from the trial using the patients trial ID and sample date. As the samples were processed in an NHS-accredited laboratory the sample labels contained patient identifiers including the date of birth, so I had to anonymise them prior to testing in a non-NHS accredited laboratory. After vortexing each tube for 10 seconds, I transferred a 350µL aliquot of each sample to a fresh tube labelled with only the trial ID, date of original sample collection and time point prior to transferring them to The Binding Site Ltd.

The clinical study exploring the utility of MS in patients with AL amyloidosis was designed in collaboration with the National Amyloidosis Centre. Anonymised aliquots of serum were sent directly to The Binding Site Ltd for MS testing from the National Amyloidosis Centre.

I recruited the patients from UHB for the non-measurable PCD study. Whole blood samples were collected by trained phlebotomists at UHB using existing ethical approval (see section 2.3.2). I transported these samples to the Binding Site Ltd in anonymised tubes where I separated the serum. The bone marrow biopsies were performed by advanced clinic nurse practitioners and trust grade doctors at UHB. SPE, IFE and serum FLC analysis were performed as part of their routine clinical care by the Immunology department at UHB. The biopsy samples were processed and reviewed by the histopathology department at UHB.

The samples for haemolysis screening were collected from healthy volunteers at The Binding Site Ltd by trained phlebotomists from UHB. Serum and plasma were separated at 24 hours, 48 hours and 72 hours by the Clinical Chemistry Department at The Binding Site Ltd.

I performed the MS testing on all of the samples presented in this thesis in the Biochemistry and Concept department at The Binding Site Ltd in Edgbaston, with the exception of the wide mass range analysis of A1L25 which was performed by Miss

Nicola Wright. I also prepared the dilution series used in the assay development presented in Chapter 3.

The following laboratory testing were performed by study collaborators and individuals as follows:

EuLITE

SPE, IFE and serum FLC analysis were performed as part of the trial and the results of this testing were provided in the trial database by Professor Cockwell.

Myeloma XI

SPE, IFE, serum FLC analysis and total immunoglobulin, creatinine, beta-2-microglobulin and albumin measurements were performed by the Clinical Immunology Service at the University of Birmingham

Bone marrow MRD analysis was performed by the Leeds Haematological Malignancy Diagnostic Service

PFS and OS data was provided by the Cancer Research UK Clinical Trials Unit at the University of Leeds

ALchemy

SPE, IFE, serum FLC analysis and NT-proBNP, eGFR, proteinuria, bilirubin, alkaline phosphatase (ALP) and gamma glutamyl transferase measurement results were provided by the National Amyloidosis Centre.

2.2. Sample Cohorts Used For Assay Development

The clinical cohorts used for training and assay development are summarised in Table 2.1 and are described in further detail in sections 2.2.1-2.2.5.

Table 2. 1 Sample cohorts used for assay development

Trial Name	Purpose/s of MS Testing	MS Testing	Time Points Tested
GEM CESAR	Training in assay performance, manual interpretation of MALDI-TOF MS spectra and training data sets for software development	EXENT/QIP-MS FLC-MS	Baseline Post induction Post ASCT Post consolidation
IMAJEM	Training in assay performance, manual interpretation of MALDI-TOF MS spectra and training data sets for software development	EXENT/QIP-MS FLC-MS	Baseline Post induction Post ASCT
ALLG 17	Training data set for software development Assessment of the suitability of using post induction samples for assignment of monoclonal protein isotype and *m/z* in patients as alternative for patients in whom no baseline sample is available	EXENT/QIP-MS FLC-MS	Baseline Post induction Post ASCT Post consolidation
EuLITE	To evaluate whether urine samples should be tested in addition to serum samples in the studies evaluating clinical utility	EXENT/QIP-MS FLC-MS	Baseline
Myeloma library samples	To compare the sensitivity of QIP-MS/EXENT, FLC-MS and Freelite®	EXENT/QIP-MS FLC-MS	The myeloma library samples only exist as multiple aliquots of single timepoint samples, which are usually from the time of initial diagnosis

2.2.1 GEM CESAR Trial

The GEM CESAR trial is a multicentre phase 2 study which enrolled 90 patients with high risk smouldering multiple myeloma across Spain. Patients were treated with six cycles of KRD induction chemotherapy followed by a high dose melphalan-conditioned ASCT and maintenance lenalidomide for two years. Disease status was evaluated using electrophoretic assays, serum FLC assays and NGF. The primary end point of the trial was MRD negativity after induction and ASCT (Mateos *et al*., 2021). Anonymised aliquots of serum from baseline, post induction, post ASCT and post consolidation were provided for MALDI-TOF MS testing, which I performed in the Biochemistry and Concept department at The Binding Site in Birmingham. In total, MALDI-TOF MS testing was completed on 343 samples.

The results of this MS testing were used as part of several clinical cohorts that provided training sets of data for the refinement of the software that was designed to automatically interpret the spectra for the finalised commercially available assay. The results of this testing were also used to inform the appropriate grouping of spectra for training sets as samples from patients who have undergone ASCT have markedly different appearances to presentation spectra due to the numerous oligoclonal peaks which form as part of the immune reconstitution process post ASCT. The software was designed by a commercial collaborator of The Binding Site Ltd, part of Thermo Fisher Scientific.

These samples were not included in the analysis of the clinical utility of MS as no associated data was provided.

2.2.2 IMAJEM Trial

The IMAJEM trial recruited 134 patients and evaluated the sensitivity and prognostic utility of PET-CT and MRI in patients with newly diagnosed multiple myeloma. The patients were treated with RVD induction with or without a consolidative high-dose melphalan-conditioned ASCT, followed by lenalidomide maintenance. The trial is registered at ClinicalTrials.gov (NCT01309334). Anonymised aliquots of serum were provided by the IMAJEM team and underwent MS testing. MS testing was completed on 568 samples from the IMAJEM trial in total. The results of this testing were used as one of the clinical cohorts, which provided training sets of data for the refinement of the software that was designed to automatically interpret the spectra for the finalised commercially available assay.

These samples were not included in the analysis of the clinical utility of MS as no associated data was available for me to analyse and the samples were processed with first generation chemistry beads whereas the clinical studies included in this PhD thesis were tested using second generation chemistry beads.

2.2.3 Australian Leukaemia and Lymphoma Group (ALLG) MM17 Trial

The ALLG trial MM17 trial is a phase 2 study evaluating the utility of carfilzomib, thalidomide and dexamethasone (KTd) in transplant-eligible patients with newly diagnosed multiple myeloma who achieved a suboptimal response (<PR) to first-line induction with bortezomib-based chemotherapy or disease refractory to bortezomib-based induction. All patients initially received four cycles of KTd, which was delivered in 28 day cycles. Patients in sCR post the fourth cycle of KTd proceeded directly to high dose melphalan ASCT and patients achieving <sCR received a further two cycles of KTd prior to proceeding to ASCT. At day+100 post ASCT, two further cycles of KTd were given as consolidation. Patients then proceeded to maintenance with thalidomide and dexamethasone, which was continued until 12 months post ASCT. The study is registered with the Australian and New Zealand Clinical trials registry (ACTRN12615000934549). The trial protocol was approved by the Alfred Health ethics committee and all participating patients provided written informed consent.

The trial recruited 50 patients from six sites and all available samples from baseline and day+100 post ASCT were tested by MALDI-TOF MS and FLC -MS. The results were used as a pilot study to confirm that an *m/z* difference of +/-10 for the doubly charged light chain were appropriate criteria to assign persistence of residual monoclonal protein. Baseline samples were tested to identify the isotype and *m/z* of the monoclonal protein and the results were compared to the spectra obtained from the end of induction and day+100 post ASCT.

2.2.4 EuLITE Trial

The EuLITE trial was a phase 2 randomised controlled trial, which randomised patients with dialysis dependent AKI due to biopsy proven cast nephropathy between intensive high cutoff dialysis and standard high-flux haemofiltration dialysis. 90 patients were recruited from 16 hospital sites across the United Kingdom and Germany. All patients received induction chemotherapy with bortezomib, doxorubicin and dexamethasone. The EuLITE trial is registered on the ISRCTN registry (ISRCTN45967602) (Hutchison *et al*., 2008a, Hutchison *et al*., 2019).

Baseline urine and serum samples were obtained from all participants at the end of each cycle of chemotherapy and at 3, 6 and 12 months (Hutchison *et al*., 2008a). The residual stored serum and urine samples had been transferred to The Binding SiteLtd under an MTA which was established prior to the initiation of this PhD project. Anonymised aliquots of stored serum and urine samples from the first available time point from 20 patients with paired samples were tested by MS to establish whether serum or a combination of both would provide the optimal panel for testing in the larger clinical cohorts used to assess the potential clinical utility of MS in patients with PCD.

2.2.5 The Binding Site Ltd Myeloma Library Samples

The myeloma library samples are anonymised serum samples which have been obtained as anonymised discard materials following routine diagnostic testing and/or procedures such as apheresis at consenting NHS laboratories across the United Kingdom. SPE, IFE and Freelite® analysis are performed on all samples by the Clinical Chemistry department at The Binding Site Ltd. The samples are divided into 0.5ml aliquots which are available for assay development work and verification testing upon request.

2.3 Clinical Cohorts used For Evaluation of Clinical Utility of the MS Assays

The clinical cohorts used for training and assay development are summarised in Table 2.2 and are described in further detail in sections 2.3.1-2.3.3.

The carfilzomib, cyclophosphamide, lenalidomide and dexamethasone (KCRD) arm was chosen as this arm of the trial had the best responses rates (Jackson *et al*., 2021) and KCRD represents the most contemporary treatment regimen included in the Myeloma XI trial.

Baseline samples were tested in all cohorts to determine the isotype and *m/z* of the monoclonal protein that was tracked in follow-up samples. Samples from post-induction, day+100 post ASCT and six months post maintenance randomisation were analysed by MS as these matched the time points where central laboratory electrophoretic, serum FLC and MRD results were available. An additional time point of 15 months post ASCT was analysed for two reasons. Firstly, it has been reported that there are significant difference in the rates of residual disease detectable by MS at day+100 in patients with different monoclonal protein isotypes, which is largely reflective of the long half-life of IgG. Secondly, the IMWG defines persistent MRD negativity as two samples analysed 12 months apart (Kumar *et al*., 2016), so the 15 months post ASCT time point provided a time point for MS analysis that was 12 months post the day+100 post ASCT analysis.

Owing to the high sensitivity of MS assays they may have particular utility in patients with PCD that are associated with low-level monoclonal protein production. Therefore all of the patients with non-measurable myeloma (monoclonal protein <10 g/L, iFLC <100mg/L and BJP <200mg/24 hours) from Myeloma XI with sufficient residual serum for MS testing were evaluated. All available samples between presentation and day+100 post ASCT were tested. The UHB cohort was included so that the agreement between the monoclonal proteins identified by MS and the immunohistochemistry results could be assessed as no histology results for the Myeloma XI samples from patients with non-measurable myeloma.

AL amyloidosis samples were selected for evaluation as it is a condition associated with lower levels of monoclonal protein production on average and there is very little data on the utility of MS assays in this patient population. In addition to the baseline samples, samples from six months and twelve months were analysed. The latter time point was important as organ responses typically take longer to achieve than haematological responses (Muchtar *et al*., 2018, Kastritis *et al*., 2021a).

Table 2. 2 Summary of the clinical cohorts used to assess the clinical utility of the MS assays in this thesis

Trial Name	Purpose/s of MS Testing	MS Testing Performed	Time Points Tested
Myeloma XI KCRD cohort	To evaluate the sensitivity of MS in comparison to electrophoretic techniques, serum FLC assays and bone marrow based MRD. To evaluate the prognostic significance of MALDI-TOF MS based assessments in comparison to IFE and bone-marrow based MRD assessments by flow cytometry	EXENT/QIP-MS FLC-MS	Baseline Post induction Day+100 post ASCT 6 months post maintenance randomization 15 months post ASCT
Myeloma XI non-measurable myeloma cohort	To evaluate the sensitivity of MS in comparison to electrophoretic techniques and serum FLC assays in patients with serologically non-measurable myeloma	EXENT/QIP-MS FLC-MS LC-MS	Baseline Post cycle 1 Post induction, Day+42 post ASCT Day+100 post ASCT
Alchemy	To evaluate the sensitivity of FLC-MS in comparison to electrophoretic techniques and serum FLC assays in patients with AL amyloidosis	FLC-MS	Baseline, 6 months and 12 months

2.3.1 Myeloma XI Trial

The Myeloma XI trial is a large phase III randomised controlled trial of patients with newly diagnosed multiple myeloma. The trial recruited 4420 patients across hospital sites in the United Kingdom between 2010 and 2016. The trial initially compared two triplet induction combinations – lenalidomide, cyclophosphamide and dexamethasone (RCD) and cyclophosphamide, thalidomide and dexamethasone (CTD). RCD was delivered in 28 day cycles and CTD was administered in 21 day cycles. Non-transplant eligible patients received attenuated CTD (CTDa) in 28-day cycles. An additional arm using the quadruplet combination of KCRD delivered in 28 days cycles was added in 2013.

Patients achieving <VGPR underwent an additional randomisation between VCD versus no additional therapy. Transplant-eligible patients then proceeded to high-dose melphalan ASCT (melphalan dosed at 200 mg/m2 for patients with a serum creatinine <200μmol/L and melphalan 140 mg/m^2 for patients with serum creatinine ≥200μmol/L). All patients then underwent a maintenance randomisation, which compared continuous lenalidomide maintenance at 10mg for days 1-21 of each 28 day cycle (with or without vorinostat 300 mg on days 1-7 and 15-21) until disease progression or intolerance versus observation (Jackson *et al*., 2019).

Responses was assessed centrally using SPE, IFE and serum FLC assessments at the following time points:

- post cycle one of induction chemotherapy
- the end of induction chemotherapy
- day+42 post ASCT
- day+100 post ASCT
- six months post maintenance randomisation
- 2-3 monthly until disease progression.

Centralised response assessments were reviewed by an expert panel and responses were categorised according to the IMWG uniform response criteria (Table 2.3). Patients who fulfilled the serological criteria for a CR but did not have a confirmatory bone marrow result were described as being in nCR (Jackson *et al*., 2021). The

centrally assigned response assessments were used for comparison against the response assessments obtained using MALDI-TOF MS.

Table 2. 3 IMWG uniform response criteria (Kumar *et al*., 2016)

IMWG Response	Criteria
Stringent complete response (sCR)	CR as defined below and a normal FLC ratio and no clonal plasma cells in the bone marrow
Complete response (CR)	Negative serum and urine IFE, disappearance of any soft tissue plasmacytomas and <5% plasma cells on bone marrow biopsy
Very good partial response (VGPR)	>90% reduction in the serum monoclonal protein and urine monoclonal protein <100 mg/24 hours or monoclonal protein detectable by serum and/or urine IFE but not by electrophoresis
Partial response (PR)	>50% decrease in the size of the serum monoclonal protein and a >90% reduction in the BJP concentration or a reduction to <200 mg/24 hours. If the serum and urine monoclonal protein concentrations are not measurable by electrophoresis, the difference between the iFLC and uFLC levels should have decreased by >50% For patients in whom there is no measurable disease in the serum and urine: >50% reduction in the percentage of plasma cells is required, provided the plasma cell percentage was >30% at presentation If there were any plasmacytomas were present at baseline, they should have reduced in size by >50%
Progressive disease (PD)	>25% increase from the maximum response in any of the following: -serum monoclonal protein (increase must be >5 g/L) -BJP (Increase must be >200 mg/24 hours) -difference between the iFLC and the uFLC and this must be >100 mg/L (only to be used for patients with multiple myeloma where the only measurable component is the serum FLC -plasma cell infiltration in the bone marrow (must be >10%) -development of new bone lesions or an increase in the size of existing bone lesions or plasmacytomas (increase must be ≥1cm)
No change (NC)	Does not fulfil the criteria for CR, VGPR, PR or PD
Clinical Relapse	Development of new end-organ damage (hypercalcaemia >2.65 mmol/L, fall in the haemoglobin level by >20 g/L or an increase in the serum creatinine to ≥ 177 µmol/L)

Age, gender and creatinine results were used to calculate the estimated glomerular filtration rates (eGFR) using the 2021-CKD EPI creatinine equation (Inker *et al*., 2021). This was done to facilitate accurate interpretation of serum FLC levels and serum FLC ratio results.

Samples were sent for centralised MRD assessment at: the end of induction chemotherapy; day+100 post ASCT; and six months post maintenance randomisation. Bone marrow MRD assessments were performed by the Leeds Haematological Malignancy Diagnostic Service using a 6-colour flow cytometry panel (CD138, CD38, CD45, CD19, CD56, and CD27). CD81 and CD117 were used in addition in some cases to provide further markers to differentiate between normal and neoplastic plasma cells. CD81 expression occurs in 100% of normal plasma cells but approximately 55-60% of neoplastic plasma cells are negative for CD81 (Andy *et al*., 2008, Paiva *et al*., 2012, Chen *et al*., 2018b). CD117 is not expressed on normal plasma cells but is expressed in 36-40% of neoplastic plasma cells (Andy *et al*., 2008, Chen *et al*., 2018b). A minimum of 500, 000 cell events were acquired, providing a minimum sensitivity of 4×10^{-5} (de Tute *et al*., 2016).

Baseline clinical and laboratory data regarding the disease burden and degree of end-organ damage that were conducted locally were collected at trial enrolment and stored centrally by the Cancer Research UK Clinical Trials Unit in Leeds. Baseline assessments included:

- Haemoglobin level and platelet count
- LDH
- Beta-2-mciroglobulin level
- Serum albumin
- The presence of bone disease
- Percentage plasma cell infiltration in the bone marrow.

The results of the beta-2-microglobulin and albumin testing performed by the Clinical Immunology Service at the University of Birmingham were used to calculate the ISS score at presentation for each patient MS testing was completed on.

The study is registered with both the ISRCTN registry (ISRCTN49407852) and the EU Clinical Trials Register (2009-010956-93). All participants provided written informed consent for participation in the trial and were consented for secondary research projects using clinical data and stored residual serum samples. The trial was conducted in accordance with the declaration of Helsinki.

2.3.2 Non-measurable Disease Patients Cohort from University Hospitals Birmingham NHS Foundation Trust

Whole blood samples were collected from patients presenting with PCD in whom there was no measurable monoclonal protein by SPE and IFE and both serum FLC values <100 mg/L as part of the ongoing study 'A study of the pathogenesis of chronic lymphocytic leukaemia and DNA repair defects in haematological malignancies.' The study has been approved by the West Midlands Research ethics committee (reference 1-/H10206/58, IRAS project ID: 40073). The samples were prepared for MS testing as described in Section 2.4.2.

The PCD were staged as part of their routine clinical care with bone marrow aspirate and trephine biopsies and the presence of any bone disease was assessed by PET-CT.

2.3.3 Amyloidosis Samples from the ALchemy Study

Samples from baseline, 6 and 12 months post treatment initiation from patients participating in the ALchemy study were provided by Professor Wechalekar at the National Amyloidosis Centre for MS testing. The NHS institutional review board provided approval for the analysis and all patients provided written informed consent in accordance with the Declaration of Helsinki.

Baseline assessments of the clonal disease burden and organ involvement were performed as part of their routine clinical care at presentation as follows:

- SPE and serum IFE
- BJP measurement and urine IFE
- Serum FLC measurement
- Percentage plasma cell infiltration in the bone marrow
- Serum creatinine and eGFR
- Proteinuria
- BNP
- Echocardiography
- Total bilirubin and alkaline phosphatase (ALP) levels.

Organ involvement was defined using the ISA Consensus Criteria (Tables 2.4) (Gertz *et al*., 2005). Cardiac responses and renal responses were evaluated using the ISA Consensus Criteria (Table 2.5) (Gertz *et al*., 2005). The haematological responses were assessed according to the ISA Consensus Criteria for Response (Table 2.6) (Comenzo *et al*., 2012). The responses in patients presenting with low FLC burden

disease were using the low dFLC criteria proposed by Milani *et al* and Dittrich *et al* (Milani *et al*., 2017a, Dittrich *et al*., 2017).

Table 2. 4 Organ involvement in AL amyloidosis (Gertz *et al*., 2005)

Organ	Criteria
Heart	Mean wall thickness >12 mm on echocardiography with no alternative cause
Kidney	Proteinuria >0.5 g/24 hours, which should be predominantly albuminuria
Liver	Hepatomegaly – liver span >15 cm in the absence of congestive cardiac failure ALP >1.5 times upper limit of normal
Gastro-intestinal (GI) tract	Biopsy confirmed involvement associated with symptoms in keeping with GI involvement
Lung	Biopsy confirmed involvement associated with respiratory symptoms Radiological evidence of interstitial infiltration
Nerve	Peripheral: symmetrical lower extremity sensorimotor peripheral neuropathy Autonomic: gastric-emptying disorder; pseudo bowel obstruction; bladder emptying dysfunction not related to direct tissue infiltration
Joint, soft tissue and/or vascular	Macroglossia Arthropathy Claudication due to presumed vascular amyloid Myopathy with confirmatory biopsy or pseudohypertrophy Carpal tunnel syndrome

Table 2. 5 Cardiac and renal response criteria in patients with systemic AL amyloidosis (Gertz *et al*., 2005, Palladini *et al*., 2014)

Organ	Response Criteria
Kidney	≥30% reduction in proteinuria or reduction in proteinuria to <0.5 g/24 hours in the absence of renal progression Creatinine and creatinine clearance must not worsen by ≥25% from baseline Progression: ≥50% increase in proteinuria (≥1 g/24 hours) or ≥25% worsening in the serum creatinine level or creatinine clearance
Heart	>30% reduction in the NT-proBNP level and >300 ng/L decrease where baseline NT-proBNP was ≥650 ng/L * Progression: >30% and >300 ng/L increase in NT-proBNP level or >33% increase in troponin level

**New York Heart Association class responses, troponin levels and ejection fraction criteria were not evaluated as these results were not available*

Table 2. 6 Haematological Response Criteria in Systemic AL Amyloidosis (Gertz *et al*., 2005, Dittrich *et al*., 2017, Milani *et al*., 2017a)

Response Category	Criteria
CR	Negative serum and urine IFE, and a normal serum FLC ratio (if skewed toward the uFLC this should be classified as a normal serum FLC ratio)
VGPR	Reduction in the dFLC to <40 mg/L (dFLC must be ≥50 mg/L at presentation)
PR	>50% reduction in the dFLC (dFLC must be ≥50 mg/L at presentation)
Low dFLC PR	Reduction in dFLC to <10 mg/L for patients presenting with dFLC >20 mg/L but < 50 mg/L
No response (NR)	Does not fulfil the criteria for CR, VGPR, PR or PD
PD	For patients in CR: re-appearance of detectable monoclonal protein or an abnormal FLC ratio (FLC must have doubled) Progression from PR or NR: ≥50% increase in the serum monoclonal protein to >5 g/L or ≥50% increase in the urine monoclonal protein to >200 mg/24 hours with a visible monoclonal peak present ≥50% increase in the iFLC with an iFLC level ≥100 mg/L

2.4 Preparation of Serum Samples for Testing by MS

2.4.1 Clinical Trial Samples

Aliquots of residual serum after completion of central laboratory testing were collected and stored at -20°C in preparation for MS analysis. Samples were thawed and vortexed for 10 seconds prior to MS testing. Samples were stored at 4°C between EXENT/QIP-MS (system and assay in development) and FLC-MS analysis to avoid repeated freeze thaw cycles.

2.4.2 Samples from Patients Treated at University Hospitals Birmingham NHS Foundation Trust with Non-measurable PCD

Blood samples from consenting patients were collected into BD Vacutainer Plus Plastic Serum tubes (Beckton Dickinson). To separate the serum whole blood was centrifuged at 1000g for 10 minutes, then transferred to fresh 2 ml screw cap tubes. Samples that were not immediately analysed were aliquoted and booked into the Clinical Chemistry Department at The Binding Site part of Thermo Fisher Scientific where they were stored at -20°C until the MS testing was performed.

2.5 Serum FLC Assessments

Samples for sensitivity analysis comparisons between FLC-MS and serum FLC assessments were prepared by diluting stored library samples from patients with FLC only secreting PCD into polyclonal serum, which had been manufactured by The Binding Site Ltd part of Thermo Fisher Scientific using pooled serum from heathy blood donors purchased from the NHS Blood and Transfusion Service. Serum FLC measurements were performed using the Freelite® assay (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom) on an Optilite analyser. The FLC testing was performed by Miss Sophie Booth from the Biochemistry and Concept team at The Binding Site Ltd part of Thermo Fisher Scientific.

All other samples had their serum FLC assessments performed as part of their routine clinical care and/or clinical trial and the results were provided as part of the response databases.

2.6 Quantitative Immunoprecipitation MALDI-TOF MS Testing (QIP-MS)

Serum samples were diluted 1:10 volume by volume (v/v) with phosphate buffered saline containing 0.1% TWEEN-20 (PBST) (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom, part of Thermo Fisher Scientific). Urine samples were diluted 1:2 v/v with PBST (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom). 100µL diluted serum per well was incubated with antisera specific for IgG, IgA, IgM, total lambda and total kappa light chains that had been conjugated to magnetic microparticles (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom) for 10 minutes in 96 well u-bottom plates (Greiner Bio-One GmbH, Austria). Urine samples were only incubated with antisera specific for total lambda and total kappa light chains that had been conjugated to magnetic microparticles (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom). Samples were washed three times with PBST and once with de-ionised water to remove non-immunoglobulin proteins. The samples were incubated with 35µL of 5% acetic acid in 20 mmol TCEP (tris(2-carboxyethyl)phosphine) (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom) to elute and reduce the bound immunoglobulins as the assay specifically tracks the light chain portion of the monoclonal protein. 1 µL eluates were co-aspirated with 1 µL of α-cyano-4-hydroxycinnamic acid (HCCA) (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom) in 1:1 v/v acetonitrile and 0.1 % formic acid aq. (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom) and spotted onto disposable Bruker target plates (Bruker, Germany). The MALDI-TOF mass spectrometer was calibrated prior to the acquisition of each target plate using Protein calibration mix 2 (ProMix2) 8,000-24,000 Da (LaserBio Labs, France). Calibration is performed prior to all mass spectrometry analysis as subtle changes in conditions such as the electronics and temperature, can affect the ability of the mass

spectrometer's ability to produce an accurate *m/z* result. A calibration mix with constituents which cover the mass range that is being analysed is chosen and the *m/z* values obtained on the calibration spot on each plate are compared to the expected values for the proteins contained within the calibration mix. ProMix2 is used to provide a five point calibration by using peaks from the singly and doubly charged ions of horse heart cytochrome C and the singly and doubly charged ions from trypsinogen (Figure 2.1). The calibration results are then applied to each mass spectrum obtained from that target plate.

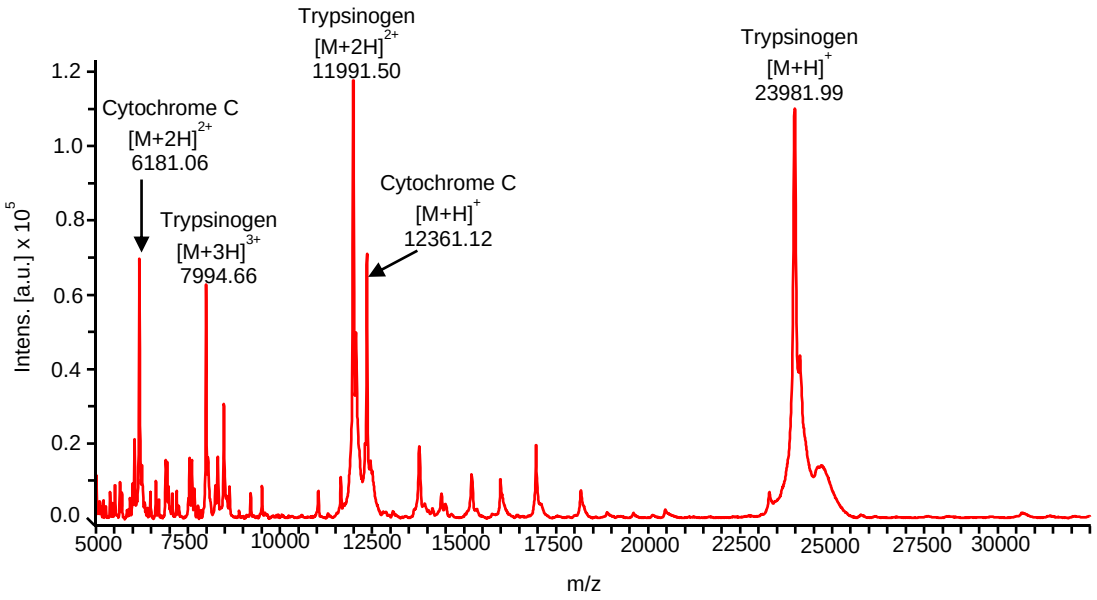


Figure 2. 1 Example mass spectrum obtained using ProMix2 for Calibration
Calibration is performed for each plate of samples prepared using ProMix2 calibration mix. The five peaks (from left to right: doubly charged cytochrome C, triply charged trypsinogen, doubly charged trypsinogen, singly charged cytochrome C and singly charged trypsinogen) are labelled with their charge state and m/z.

2000 summated shots per spot were acquired using a microflex MALDI-TOF mass spectrometer (Bruker, Germany) in a positive ion mode over an *m/z* range of 5000-32000. Example mass spectra over the *m/z* range 5000-32000 for a polyclonal sample are shown in Figure 2.2. Mass spectra focusing on the doubly charged light chain

region showing the typical appearances of a polyclonal sample in comparison to sample containing a monoclonal protein are shown in Figure 2.3.

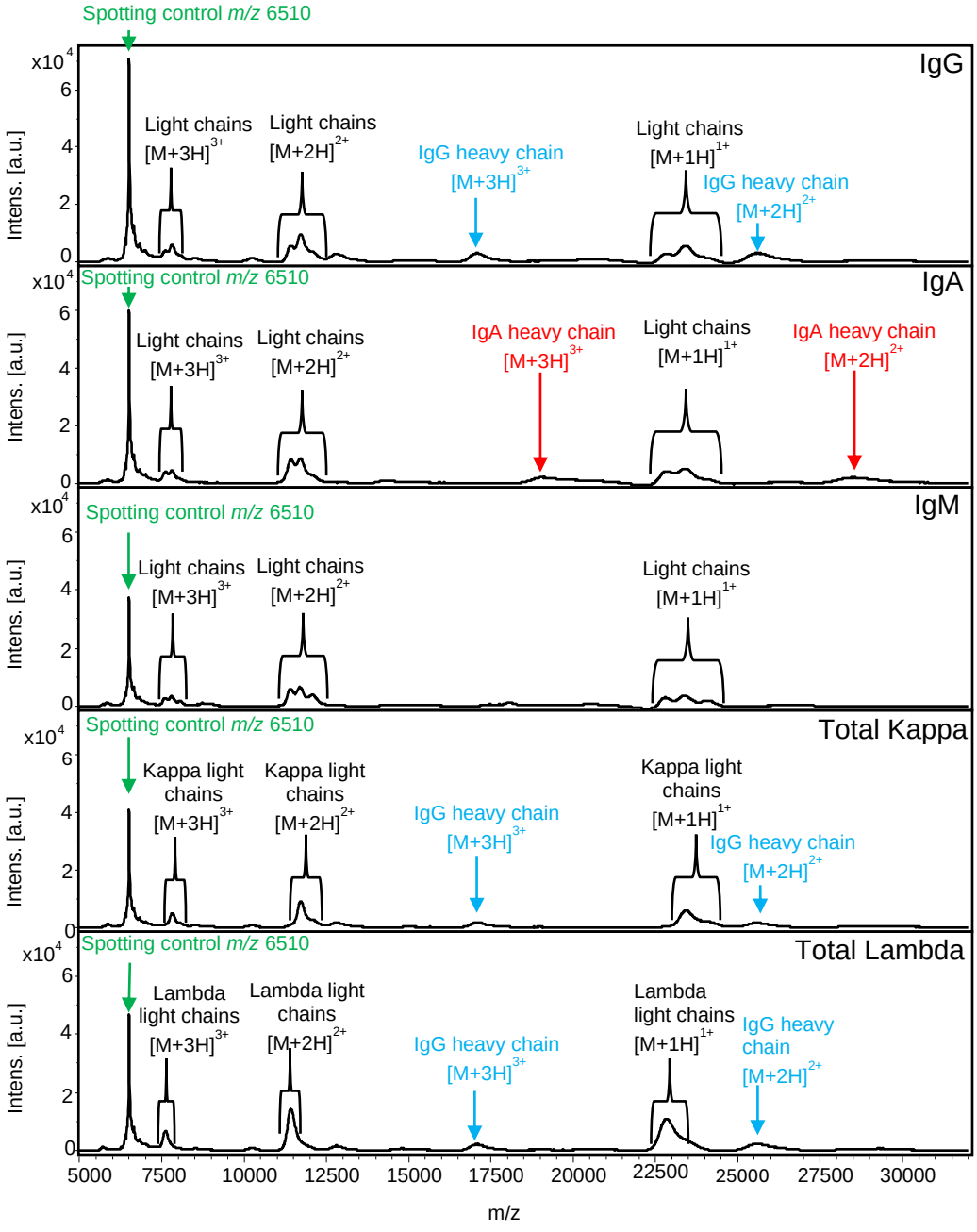


Figure 2. 2 Typical appearances for mass spectra for a polyclonal sample obtained using the QIP-MS/EXENT assay.
The m/z regions in which the singly, doubly and triply charged light chains are highlighted with black brackets in each mass spectrum. In the IgG, total kappa and total lambda spectra doubly and triply charged IgG heavy chains can also be seen (highlighted by the blue arrows in the relevant spectra) at a lower intensity than the light chain peaks as the assay conditions are optimised for the assessment of the light chain portion of immunoglobulins. In the IgA mass spectrum doubly and triply charged IgA heavy chains can also be seen (highlighted by the red arrows in the IgA spectrum).

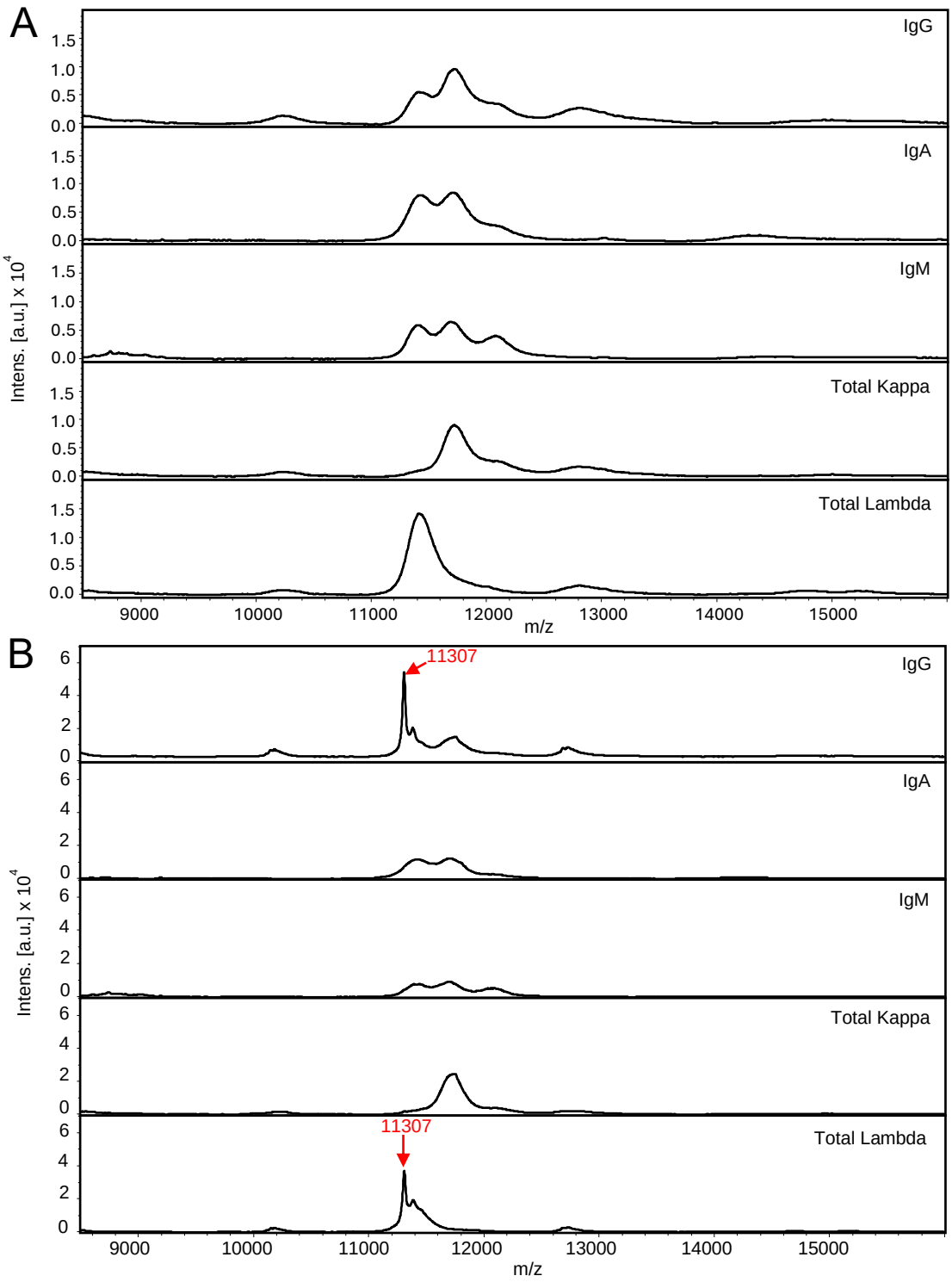


Figure 2.3 Monoclonal proteins appear as sharp peaks above the polyclonal background with a specific isotype and *m/z*

The typical appearances for the doubly charged light chain peaks from a polyclonal sample tested by QIP-MS/EXENT are shown in (A). Doubly charged lambda and kappa light chains are found between m/z 11168 and m/z 11540 and m/z 11540 and m/z 12401 respectively. (B) Shows the doubly charged light chain regions for a sample containing an IgG lambda monoclonal protein. The monoclonal protein is easily identifiable as a sharp peak above the polyclonal background at m/z 11307 for the doubly charged light chain in the spectra obtained from the IgG and lambda eluates and is highlighted by the red arrows and mass labels.

Mass spectra were assessed as containing spectral patterns consistent with N-linked glycosylation if polytypic peaks with *m/z* differences consistent with typical N-linked glycans were observed between *m/z* 12300 and *m/z* 13500 for the doubly charged light chains and *m/z* 24600 and *m/z* 27000 for the singly charged light chains (Figure 2.4).

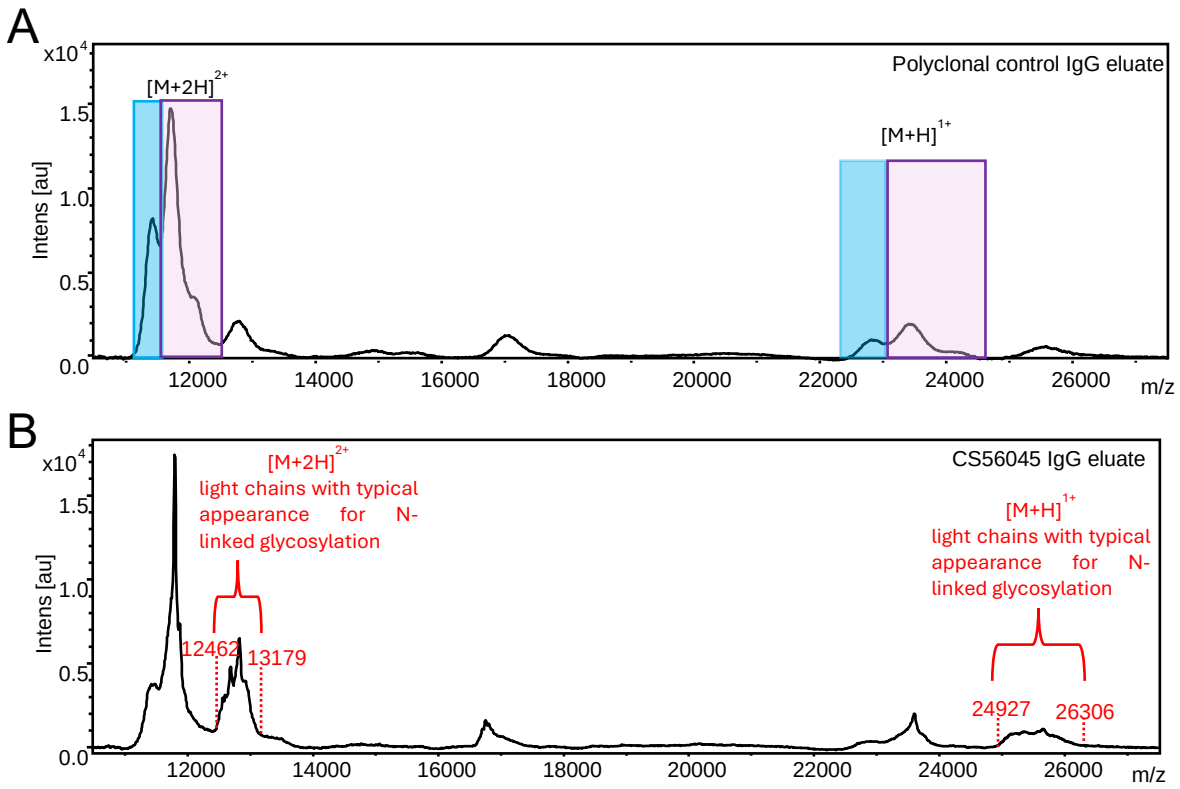


Figure 2.4 Identification of light chain N-linked glycosylation in MALDI-TOF MS spectra

The standard m/z regions for non-glycosylated lambda and kappa lights chains are highlighted by the blue and purple boxes in (A). Non-glycosylated lambda light chains are typically found between m/z 11168 and 11540 for the doubly charged light chains and m/z 22330 and 23078 for the singly charged light chains. Non-glycosylated kappa light chains are typically found between m/z 11540 and 12401 for doubly charged light chains and m/z 23078 and 24650 for singly charged light chains. Spectral patterns consistent with light chain glycosylation are identified by the presence of "hedgehog" shaped peaks found to the right of the non-glycosylated light chain m/z regions due to their higher m/z. The singly and doubly charged glycosylated light chains in a patient with an IgG kappa monoclonal protein are highlighted by the red brackets and mass labels in (B). The doubly charged glycosylated light chains of the monoclonal protein in this sample are found between m/z 12462 and m/z 13179. The same peaks were also present in the corresponding total kappa spectrum from this patient (mass spectrum not shown).

Mass spectra were analysed blinded to the central laboratory response assessment results. Spectra were initially visually inspected and scored categorically as positive or negative. Follow-up spectra were scored as positive if there was a residual monoclonal peak with the same isotype as that detected in the baseline sample and within *m/z* +/-10 of the monoclonal peak identified at baseline.

Once the prototype software development had been completed the spectra were run through the software for quantitation. Monoclonal protein peaks were quantitated by multiplying the percentage of the total area of the doubly charged light chain region that was represented by the monoclonal peak by the nephelometry value for the relevant immunoglobulin isotype using a prototype version of the EXENTiQ software (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom). This five bead assay is trademarked as the EXENT assay (system and assay under development) (Ashby JA, 2018).

2.7 FLC MALDI-TOF MS Testing

Serum samples were diluted (1:2 (v/v) for samples from patients with systemic AL amyloidosis and 1:4 (v/v) for samples from patients with multiple myeloma) with PBST (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom). Urine samples were diluted 1:2 v/v in PBST. 100µL diluted serum or urine was incubated with antisera specific for kappa and lambda FLC that had been conjugated to magnetic microparticles for 30 minutes in 96 well u-bottom plates (Greiner Bio-One GmbH, Austria). Samples were then washed three times with PBST and twice with de-ionised water. The bound FLC were then eluted and reduced with the addition of 35µL of 5%

acetic acid with TCEP (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom) for 15 minutes. 1 µL eluates were co-aspirated with 1 µL of HCCA in 1:1 (v/v) acetonitrile and 0.1 % formic acid (aq.) and spotted onto disposable Bruker target plates (Bruker, Germany). Positive ion mode spectra were acquired using a microflex MALDI-TOF mass spectrometer (Bruker, Germany) over a mass range of 5000 – 32000 Da. The mass spectrometer was calibrated prior to the acquisition of each target plate using ProMix2 (LaserBio Labs, France). Mass spectra were analysed manually using FlexAnalysis (Bruker, Germany).

2.8 LC-MS testing

Samples underwent immune precipitation using the sample protocol used to prepare samples for MALDI-TOF MS testing as described in Sections 2.6 and 2.7. Reversed phase LC-MS analysis was performed on a Waters ACQUITY I Class Ultra-High Performance Liquid Chromatography system coupled to a Xevo G2-XS hybrid quadrupole-time of flight mass spectrometer (Waters, United Kingdom). 20 µL of the eluates from the immunoprecipitation stage were transferred into total recovery autosampler vials (Waters, United Kingdom). 10 µL was injected on to a BioResolve RP mAb Polyphenyl column [450A, 2.7 µm, 1.0 x 150 mm] (Waters, United Kingdom) and separated with a gradient consisting of mobile phase A (0.1% formic Acid in water) and mobile phase B (0.1% Formic Acid in acetonitrile) at a flow rate of 0.2 mL per minute flow rate. The chromatography gradient was as follows: the organic mobile phase B was set to 20% at 0 minutes and the organic phase was increased to 55% over 20 minutes. Mobile phase B was then increased to 80% over 0.3 minutes and maintained at 80% for a further 1 minute. Mobile phase B was then returned to 20% over 0.3 minutes and maintained at 20% for a further 3.4 minutes for re-equilibration.

The total run time including column conditioning was 25 minutes. The sample manager temperature was set to 4 °C and the column temperature was set to 80 °C. Positive electrospray ionisation mode was used, and the source parameters were set as follows: capillary voltage 1.5 kV; sample cone voltage 110 V; source temperature 120 °C; desolvation temperature 450 °C; cone gas flow 10 L per hour; and desolvation gas flow 600 L per hour. Intact protein analysis was performed by scanning a mass range of 500-4000 *m/z*. MS data were acquired over 25 minutes scanning between 500-4000 *m/z*. LockSpray was enabled and Leucine Enkephalin was measured for 0.25 seconds every minute at 3kV capillary voltage and 30 V cone voltage.

For each sample, a summation of the mass spectra was generated from the relevant retention time window (between approximately 6.5 minutes and 7.5 minutes) where the light chain was expected to have eluted. The mass spectrum was smoothed using Savitzky-Golay settings of 1 data point for half width and 1 iteration. Baseline subtraction was then applied, and the spectra were deconvoluted using the automatic settings within UNIFI (Waters, United Kingdom) using the MaxEnt1 algorithm. This algorithm works by deconvoluting the summed raw multiple charge state data within the specified time window of the total ion chromatogram into the most likely singly charged mass spectrum (Ferrige *et al*., 1992).

Figure 2.5 shows a total ion chromatogram and deconvoluted mass spectrum of a sample containing a monoclonal protein, which appears as a sharp high intensity peak.

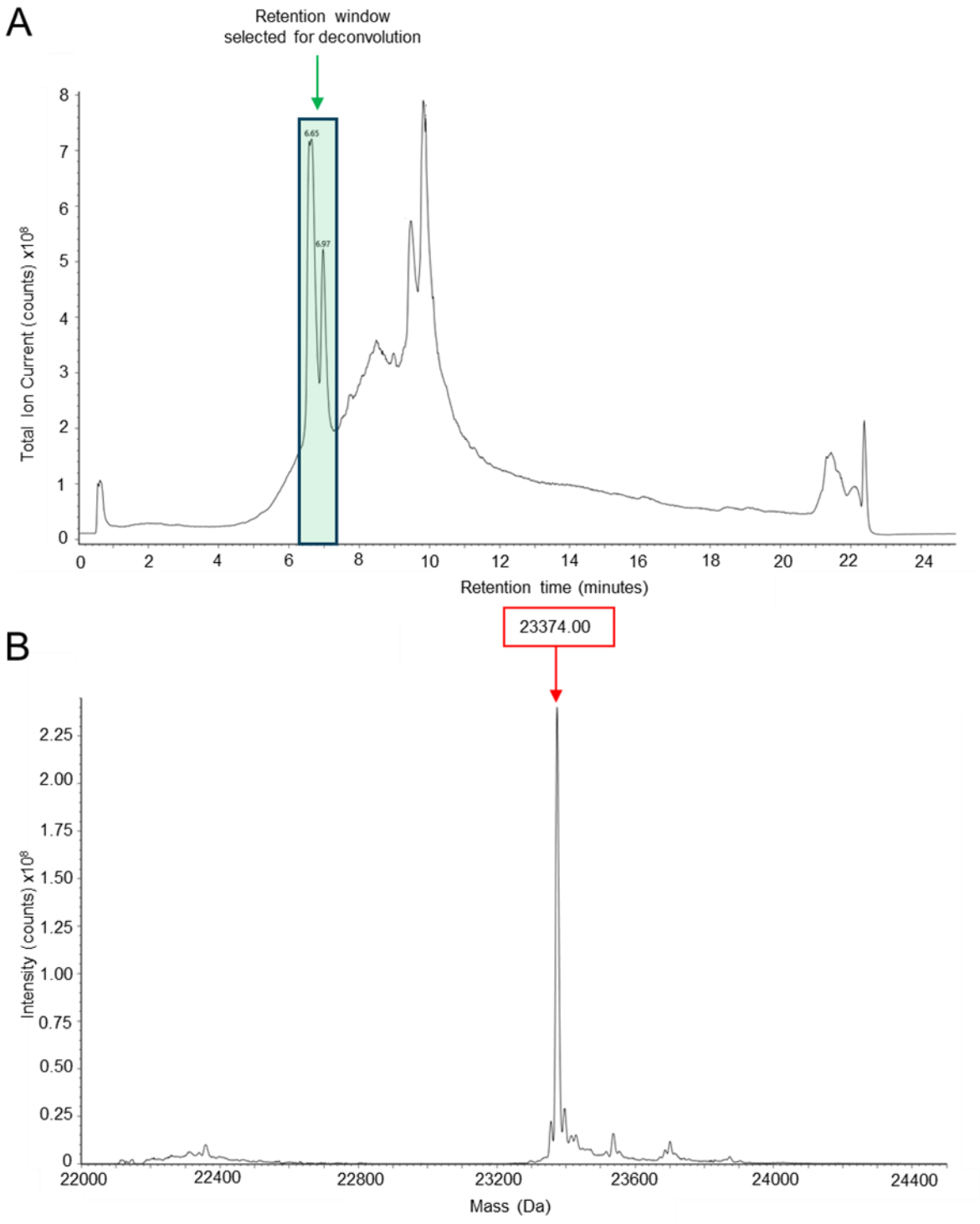


Figure 2.5 Analysis of LC-MS data

(A) Shows an example total ion chromatogram with the retention time window between 6.5 and 7.5 minutes (highlighted by the green box and arrow) selected for deconvolution. (B) Shows the deconvoluted mass spectra from the 6.5-7.5 minute retention window, with the mass label for the monoclonal light chain identified, which has an m/z of 23374 for the singly charged light chain and is highlighted by the red arrow. The other smaller peaks in the background of this mass spectrum represent the polyclonal background, which is relatively suppressed (total IgG 8.53 g/L, IgG kappa monoclonal protein 4.34 g/L).

2.8.De-glycosylation of Monoclonal FLC

Serum samples were diluted 1:4 with PBST (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom, part of Thermo Fisher Scientific) and incubated with antisera specific for the relevant immunoglobulin isotypes conjugated to magnetic microparticles (The Binding Site Ltd part of Thermo Fisher Scientific, United kingdom) for 30 minutes. Samples were washed three times with PBST (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom) and once with standard de-ionised water. The immobilised immunoglobins and paramagnetic particles were resuspended in 100 μL of de-ionised water and incubated with 2 μL of Peptide *N*-glycosidase F (PNGase F) (Promega, United Kingdom and Roche, Switzerland) at 37 °C for 24-72 hours with continuous rotation at 1000 rpm. Samples de-glycosylated using PNGase F supplied by Promega required an additional wash step with 50 μL of 20 mM EDTA prior to resuspension in de-ionised water and addition of the PNGase F.

Additional de-glycosylation protocols were explored but less successful and are described in Chapter 3.

The tubes were then placed in a magnetic rack for 60 seconds to pellet the magnetic microparticles and the supernatant was removed. Captured FLC were eluted with 35μL 5% acetic acid with 20 mmol TCEP (The Binding Site Ltd part of Thermo Fisher Scientific, United Kingdom, part of Thermo Fisher Scientific) for 15 minutes. MALDI spotting and acquisition was performed as described in Section 2.6.

2.9. Statistical Analysis

In the Myeloma XI trial, the primary outcome being assessed in the patients randomised between KCRD versus the triplet combinations of RCD and CTD are PFS and OS. In 2021 the PFS results of this arm of the trial were published and showed that KCRD resulted in improved PFS compared to triplet therapy. Follow-up is ongoing for the co-primary endpoint of OS and will be analysed once 50% of patients have died. PFS was calculated from the time of randomisation to the time of disease progression or death or last follow-up. OS was calculated as the time from randomisation to death or last follow-up (Jackson *et al*., 2021). The wider Myeloma XI trial also evaluated the effectiveness of lenalidomide maintenance. Two primary outcome measures were assessed in the patients who underwent this randomisation – PFS and OS. These were both calculated from the time of randomisation between lenalidomide continuously until progression and observation (Jackson *et al*., 2019). The primary objective of the ancillary study performed as part of this PhD thesis was to assess whether achieving MS-negativity was associated with improved PFS. The secondary objectives were: to compare the sensitivity of MS compared to electrophoretic assay and serum FLC assessments; to compare agreement between bone marrow MRD assessed by flow cytometry and MS-based assessments; to assess whether MS-negativity was associated with improved PFS in patients with no residual detectable disease by serum IFE; and to assess the prognostic significance of light chain N-linked glycosylation. The relationship between OS and MS-assessed responses will be re-assessed once 50% of the patients included in this ancillary study have died.

Owing to the relatively small number of patients with samples available for MS testing with non-measurable myeloma the primary objective of this study was to compare the rate of monoclonal protection by MS to the electrophoretic techniques and serum FLC assays but no survival analysis was planned.

ALchemy is an ongoing real-world study of chemotherapy prescribing and outcomes for patients being treated for AL amyloidosis in the United Kingdom. The initial aim of the study was to evaluate the impact of intensifying follow-up with three monthly rather six-monthly follow-up assessment by the National Amyloidosis Center and collecting monthly outcome data regarding chemotherapy and adverse effects from the local physicians. The primary aim of the ancillary study performed using samples from the ALchemy study for this PhD was to assess whether FLC-MS negativity was associated with improved OS in patients undergoing treatment for AL amyloidosis. OS was calculated from initiation of chemotherapy for AL amyloidosis to the time of death or last follow-up. The secondary objectives were: to compare the sensitivity of FLC-MS to SPE, IFE and serum FLC assessments in patients with newly diagnosed AL amyloidosis and at follow-up during treatment; to assess whether MS-negativity was associated with improved renal and cardiac response rates; and to assess the prognostic significance of FLC glycosylation in patients undergoing treatment for AL amyloidosis.

Statistical analysis was performed using SPSS 28.0. PFS was assessed using the Kaplan-Meier method and the log-rank test was used to assess the statistical significance of differences between the survival curves. Median follow-up was

calculated using the reverse Kaplan-Meier method. Hazard ratios were calculated using the Cox proportional hazards model alongside 95% confidence intervals. Univariate and multivariate analysis were performed using the Cox proportional hazards method.

Agreement between quantitative results obtained by SPE and MALDI-TOF MS were compared using intraclass correlation (ICC) analysis using a two-way mixed model evaluating absolute agreement between values obtained by the two methods.

The statistical difference between categorical variables were compared using the Chi squared test. Yates correction was applied to results obtained from 2 x 2 contingency tables.

The normality of distribution for continuous variables was assessed using histograms for samples cohorts >100. The Shapiro-Wilk test was used for sample cohorts <100 and samples were assumed to follow a normal distribution if the p value was > 0.05. Differences between normally distributed continuous variables were assessed using the student's t-test and the difference between non-normally distributed continuous variables were assessed using Mann-Whitney U-tests.

For all statistical analyses performed p values <0.05 were considered statistically significant.

3: MASS SPECTROMETRY ASSAY DEVELOPMENT

3.1 Introduction

The Mayo clinic were the pioneers of using intact light chain MS-based assays for the detection and monitoring of monoclonal proteins in patients with PCD, with their in-house developed MASS-FIX assay replacing IFE in their laboratory during 2018 (Kohlhagen *et al*., 2020, Mellors *et al*., 2021). Although the early results of clinical studies comparing the sensitivity, throughput and prognostic significance of MASS-FIX results were encouraging (Mills *et al*., 2016, Milani *et al*., 2017b, Kohlhagen *et al*., 2020), this assay had been developed to a laboratory diagnostic test specification and is therefore only validated for use in a single institution.

In order for the MASS-FIX assay to be commercialised and sold for use as an *in vitro* diagnostic test (IVD) for monitoring patients with PCD across the globe it would need to be further developed to remove the use of reusable plates, which require harsh chemicals to clean and carry the risk of sample carry over if cleaning is inadequate. The manual elements of result analysis would also need to be minimised through further development of the software developed for the assay .

The first company develop a commercialised MS-based intact light chain assay for the detection and monitoring of monoclonal proteins in patients with PCD was The Binding Site Ltd, which is part of Thermo Fisher Scientific. The work described in this chapter details the contributions I made to the development process of the QIP-MS/EXENT

assay. This assay achieved regulatory approval for use as an IVD in Europe in August 2023. Given the commercially sensitive nature of this cutting edge technology specific details and data relating to assay reagents and equipment have not been included in this thesis as they covered by a non-disclosure agreement since their inclusion could be advantageous to other companies that may be developing technology in this field.

Regulatory approval for an IVD requires:

- defining the performance characteristics of the IVD
- the production of testing kits including performance sheets and information on storage and stability of the reagents within the testing kit
- traceability of values assigned to controls and calibrators
- all measures possible to have been taken to reduce risks associated with the incorrect identification of specimens
- adherence with good manufacturing practices and quality systems
- the elimination or maximum practicable reduction in risks associated with the use of the IVD through safe design and manufacture (FDA, 2021, EUR-Lex, 2023).

The workflow for the MASS-FIX assay is summarised in Figure 3.1.

Samples are incubated with camelid nanoparticles coated with antisera specific for IgG, IgA, IgM, total kappa and total lambda for 15 minutes in a 384 well TTP plate on a Hamilton Starlet automated liquid handler

↓

The liquid handler performs a series of washes to remove the non-immunoglobulin proteins

↓

The captured immunoglobulins are eluted with 0.1% trifluoroacetic acid and reduced with 20mM TCEP on the liquid handler

↓

The TTP plate is transferred to a TTP Labtech mosquito liquid handler which combines the samples with HCCA and spots the samples onto polished steel Bruker target plates

↓

In-house developed software imports the sample barcodes scanned by the Hamilton liquid handler.

The user scans the barcodes on the reusable polished steel MALDI plates and inserts them into the Bruker microflex mass spectrometer and the plates are analysed by MALDI-TOF MS.

↓

The software extracts the patient details from the laboratory information system and imports the five spectra generated per patient, smoothes them and displays them on three charts – IgG with total kappa and total lambda, IgA with total kappa and total lambda and IgM with total kappa and total lambda (A). The software displays the immunoglobulin results, the patient's historical MASS-FIX testing results (C) and peak statistics (D).

The spectra are manually interpreted by the user and are inserted in the interpretation field of the display generated by the software (B).

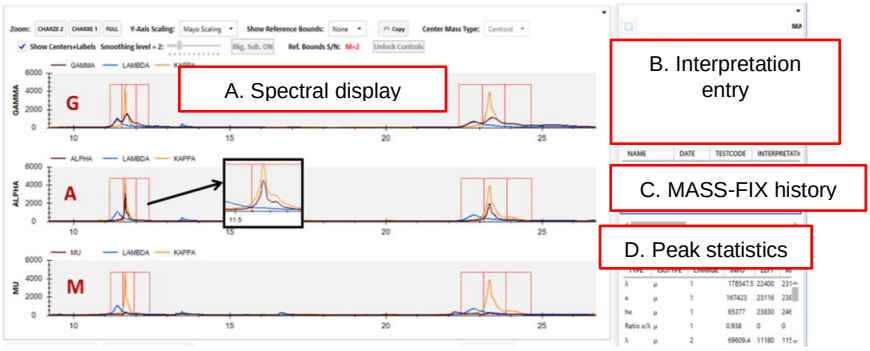


Figure 3. 1 A flow diagram of the workflow of the MASS-FIX assay.
The assay involves the use of three pieces of equipment: a Hamilton liquid handler for the immune enrichment step; a TTP Labtech mosquito liquid handler for spotting the samples onto the target plate; and a Bruker microflex MALDI-TOF mass spectrometer. The display generated by the in house software is shown at the bottom of the flow diagram. Stacking of spectra is automatically performed by the in house software (A) but the spectra are interpreted manually with results inputted in the area labelled (B). (C) shows the area where historical results are displayed and (D) shows peak statistic results.

Figure adapted from: Kohlhagen et al. Automation and validation of a MALDI-TOF MS (MASS-FIX) replacement of immunofixation electrophoresis in the clinical lab. Clin Chem Lab Med 2020; 59(1): 155-163.

Achieving regulatory approval for an IVD requires a multidisciplinary team approach, which in the case of an intact light chain MS-based assay requires:

- mass spectrometry specialists for the development of appropriate instrument settings and verification of appropriate mass spectrometry data interpretation during the development of the software for the automatic interpretation of mass spectra
- antisera procurement or developers of the antisera which will be conjugated to the capture beads/resins
- bead manufacturers who can provide large scale conjugation of reagents for the immune enrichment step and complete final stage blocking to eliminate non-specific binding of non-immunoglobulin proteins
- assay verification and validation specialists who assess the limits of conditions for use such as the storage temperature and shelf-life of the reagents and the performance characteristics as defined in the instruction for use in each test kit
- statisticians for data analysis and assistance with experimental design
- software developers
- liquid handler programmers to develop the scripts for automated sample processing and spotting
- clinical and/or laboratory collaborators for the external validation of assay performance using serial samples for patients undergoing treatment for PCD.

The aims of the work presented in this chapter were:

- Contribute to the development of an intact light chain MALDI-TOF MS based assay that will be submitted for regulatory approval

- Identify the most appropriate sample type/s for testing in the studies evaluating the potential utility of the intact light chain MALDI-TOF MS assays in patients with PCD
- Identify the optimal reagent panel for the MALDI-TOF MS testing for the clinical studies evaluating the potential utility of the intact light chain MALDI-TOF MS assays in patients with PCD
- Develop a prototype assay that could be performed confirm the presence of N-linked light chain glycosylation with the potential for automation

3.2 Results

3.2.1 MALDI-TOF MS Testing of Serum Provided Greater Sensitivity for the Detection of Monoclonal Light Chains Compared to MALDI-TOF MS Testing of Urine in Patients with Newly Diagnosed Multiple Myeloma Treated in the EuLITE Trial

20 patients from the EuLITE trial had paired urine and serum samples available for MALDI-TOF MS testing. Monoclonal light chains were detectable by MALDI-TOF MS in the serum of 20/20 (100%) using total light chain specific reagents and FLC specific reagents (Figure 3.2A). In the paired urine samples monoclonal light chains with the same isotype and *m/*z were detectable in 19/20 (95.0%) samples (Figure 3.2B). The spectra obtained from the serum and urine samples from the patient with no monoclonal light chains detectable in the baseline urine sample are shown in Figures 3.2D and 3.2E respectively. In the serum sample monoclonal lambda light chains with an *m/*z of 11441 for the doubly charged light chains were identified but no corresponding peak was seen in the lambda mass spectrum from the urine sample. However, several other peaks in the *m/z* region for the doubly charged light chains (highlighted by the red box in Figure 3.2C), which were not observed in the mass

spectrum obtained from the serum sample, were present. However, these peaks were not present in the *m/z* region for the singly charged light chains (highlighted by the green box in Figure 3.2F), suggesting that these additional peaks observed did not represent doubly charged light chains.

Overall, additional non-immunoglobulin related peaks were observed in the *m/z* region for the doubly charged light chains but not in the *m/*z region for singly charged light chains in 7/20 (35.0%) of the paired urine samples analysed (Figure 3.2C). These results suggest that additional post-incubation washes would be required to remove residual non-immunoglobulin proteins for urine samples prior to elution and reduction.

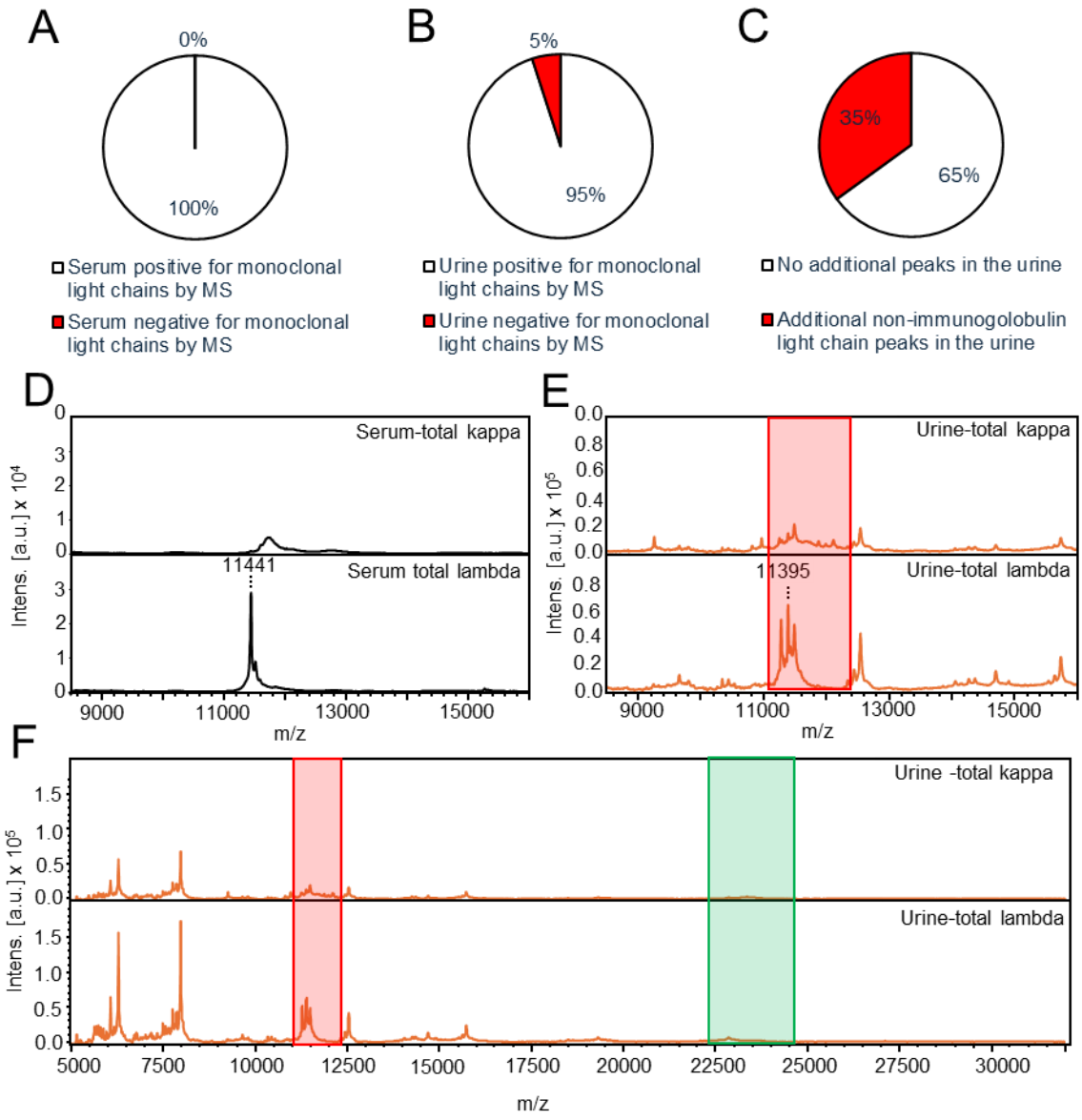


Figure 3. 2 Additional non-light chain peaks are visible in the mass spectra obtained from urine samples of patients treated in the EuLITE trial using standard EXENT assay testing conditions

The detection rate for monoclonal light chains by QIP-MS/EXENT in the serum and urine of patients treated in the EuLITE trial are shown in (A) and (B) respectively. (C) Shows the proportion of urine samples in which additional non-immunoglobulin light chains peaks were observed in the m/z region for the doubly charged light chains. (D) and (E) show the mass spectra obtained from the serum and urine of the pair of samples in which no monoclonal light chains were detectable by MS in the urine. In the urine sample from this patient several peaks are present in the m/z region for the doubly charged light chains (highlighted by the red boxes in (E) and (F)) but none of them match the m/z of the monoclonal lambda light chains detected in the serum (highlighted by the blue mass label in (C)). The additional peaks observed in urine sample in the m/z region for the doubly charged light chains (highlighted in red in (F)) were not present in the m/z region for the singly charged light chains (highlighted by the green box in (F)).

3.2.2 The Inclusion of FLC Specific Antisera in MALDI-TOF MS-based Assays Provides Greater Sensitivity for the Detection of Residual Monoclonal FLC Compared to Assays that Only Include Total light Specific Reagents

To evaluate whether a MALDI-TOF MS-based assay that included FLC specific reagents (FLC-MS) could provide greater sensitivity for the detection of low-level residual monoclonal FLC compared to QIP-MS/EXENT and Freelite®, five serum samples from patients with FLC only multiple myeloma (iFLC 114.46 – 18115.52 mg/L at presentation) were diluted into polyclonal serum from healthy blood donors and then analysed by QIP-MS/EXENT and FLC-MS. The panel of five samples used to assess the relative sensitivity of the three assays was composed of four myeloma library samples and one FLC only myeloma sample from a patient treated in the Myeloma XI trial. The samples underwent an initial dilution with PBST mixed with serum from healthy blood donors to give an iFLC concentration of 100-200 mg/L and then doubling dilutions with PBST mixed with serum from healthy blood donors were performed and the samples were tested by QIP-MS/EXENT and FLC-MS. The highest dilution at which residual monoclonal FLC was detectable by QIP-MS/EXENT and FLC-MS are shown in Table 3.1.

Residual monoclonal light chain was detectable following initial dilution in 4/5 (80.0%) of samples using QIP-MS/EXENT and 5/5 (100%) samples by FLC-MS (Table 3.1). In the sample with no detectable monoclonal light chain by QIP-MS/EXENT following initial dilution the monoclonal light had N-linked glycosylation, suggesting the heterogeneous nature of these peaks may affect assay sensitivity.

Table 3. 1 Sensitivity comparisons between QIP-MS/EXENT, FLC-MS and Freelite® for the detection of residual monoclonal FLC in samples from patients with FLC only monoclonal proteins

Samples Used to Assess Relative Sensitivity of the Three Assays	Dilution	Serum Free Kappa (mg/L)	Serum Free Lambda (mg/L)	sFLC Ratio	Sensitivity Limit
FL05 Library sample	Neat	13.38	2438.83	182.27	
FL05 Library sample	1 in 160	7.89	52.25	6.62	Highest dilution positive by EXENT
FL05 Library sample	1 in 2560	8.20	10.87	1.33	Highest dilution positive by FLC-MS
Myeloma XI FLC myeloma sample	Neat	2306.08	20.76	111.08	
Myeloma XI FLC myeloma sample	1 in 256	8.49	22.45	0.38	Highest dilution positive by EXENT
Myeloma XI FLC myeloma sample	1 in 2048	8.33	11.58	0.72	Highest dilution positive by FLC-MS
FK01 Library sample	Neat	1376.99	3.87	355.81	
FK01 Library sample	1 in 64	28.68	8.48	3.38	Highest dilution positive by EXENT
FK01 Library sample	1 in 256	12.49	9.15	1.37	Highest dilution positive by FLC-MS
FK15 Library sample	Neat	114.46	3.08	37.16	
FK15 Library sample	1 in 16	12.29	8.81	1.40	Highest dilution positive by EXENT
FK15 Library sample	1in 64	8.77	8.31	1.06	Highest dilution positive by FLC-MS
FK06 Library sample	Neat	18115.52	5.51	3287.75	
FK06 Library sample	1 in 100	not tested	not tested	not tested	Negative by EXENT
FK06 Library sample	1 in 1600	16.55	9.88	1.68	Highest dilution positive by FLC-MS

In the four samples in which residual light chain only monoclonal protein was detectable by QIP-MS/EXENT following the initial dilution, Freelite® testing was performed on the highest dilution at which residual monoclonal protein was detectable by MS to compare the sensitivity of the QIP-MS/EXENT assay to Freelite®. The results of this testing are shown in Table 3.1. In 2/4 (50.0%) of these samples residual monoclonal FLC was detectable by QIP-MS/EXENT but the serum FLC ratio and iFLC were both within the normal range. Example mass spectra from baseline and the highest dilution at which residual monoclonal FLC were detectable by QIP-MS/EXENT but no residual monoclonal FLC was detectable by Freelite® are shown in Figures 3.3A and 3.3B.

In both of the samples in which residual monoclonal FLC were detectable by Freelite® at the highest dilution at which residual light chain only monoclonal protein was detectable by QIP-MS-EXENT, the monoclonal protein had an *m/z* that sat close to the apex where the polyclonal back light chain signal intensity is highest (Figures 3.3C-F).

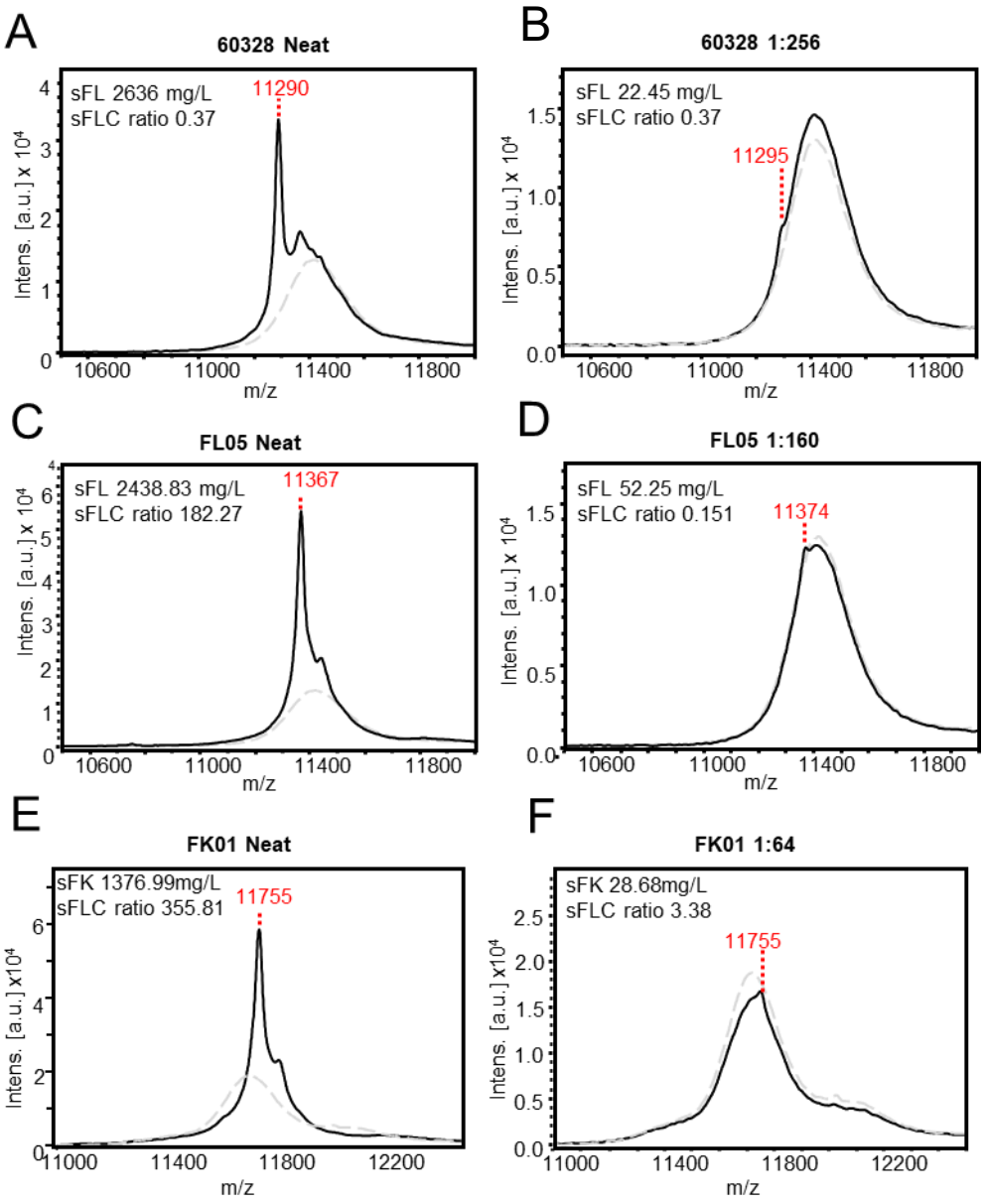


Figure 3. 3 Sensitivity of QIP-MS/EXENT versus Freelite® for the detection of residual monoclonal FLC

The baseline mass spectrum obtained from a sample tested by QIP-MSEXENT in which residual FLC monoclonal protein was detectable by QIP-MS/EXENT following 1:256 dilution into polyclonal serum but no residual disease was detectable by Freelite® is shown in (A) and the sample following 1:256 dilution is shown in (B). Mass spectra for the two samples in which QIP-MS/EXENT did not provide additional sensitivity compared to Freelite® are shown in (C-F). The monoclonal peaks are highlighted by the red dashed lines and red mass labels in each of these figures and mass spectra obtained from polyclonal control samples are shown as the grey dashed lines in each mass spectrum.

Freelite® testing was also performed on the highest dilution for each sample at which residual monoclonal FLC was detectable by FLC-MS and the results are summarised in Table 3.1. In 4/5 (80.0%) of these samples the FLC ratio had normalised and the level of the iFLC was within the normal range. Example mass spectra from one of the samples in which FLC-MS testing detected residual monoclonal FLC but the FLC ratio had normalised are shown in Figure 3.4.

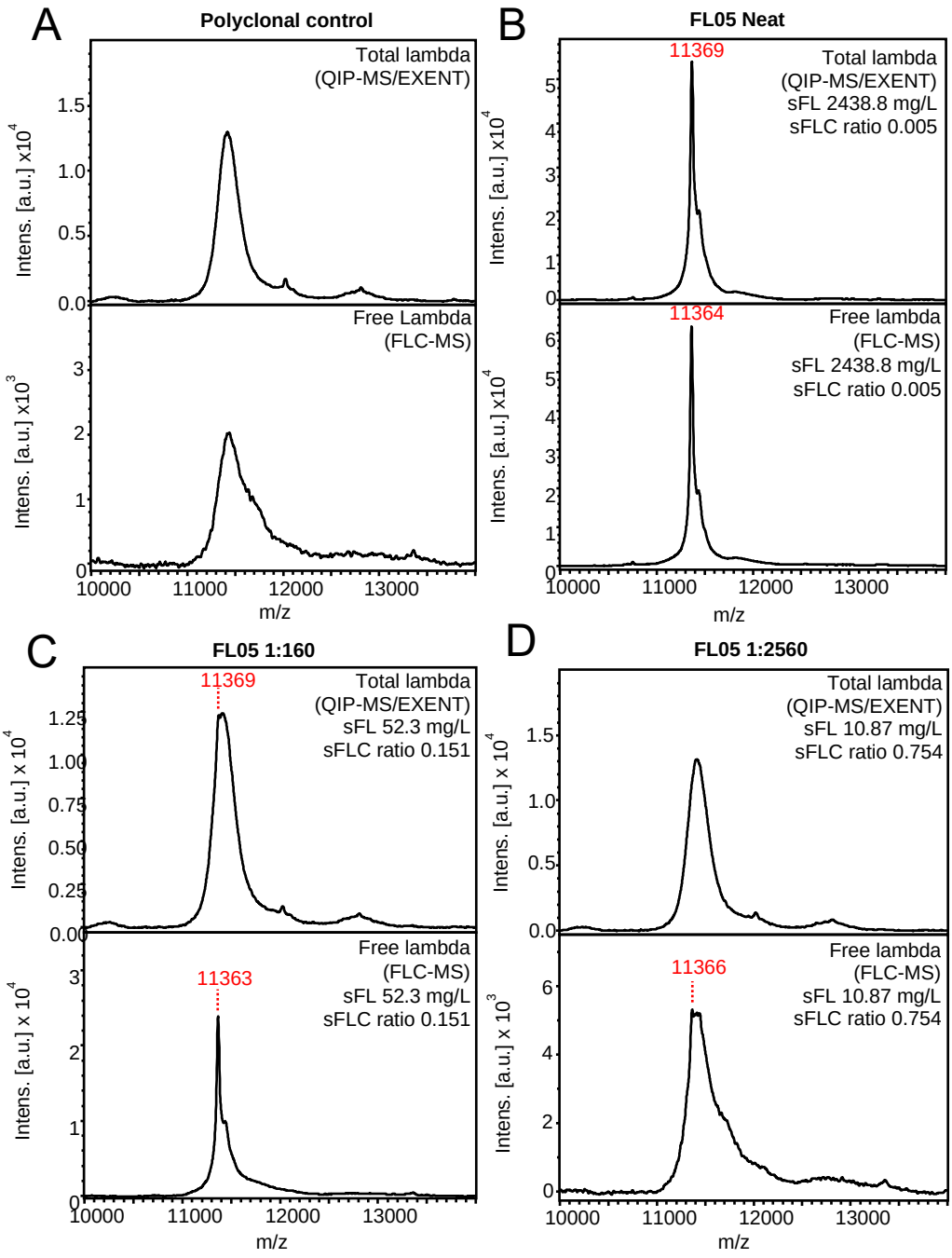


Figure 3. 4 FLC-MS provides greater sensitivity for the detection of residual monoclonal FLC compared to EXENT and Freelite®

(A) Shows example mass spectra obtained from a polyclonal sample tested by QIP-MS/EXENT with total lambda beads (top mass spectrum) and by FLC-MS with free lambda beads (bottom mass spectrum). The lambda FLC monoclonal protein identified by QIP-MS/EXENT testing and FLC-MS in the neat serum sample from the myeloma library sample FL05 are shown in (B). The highest dilution at which residual lambda FLC monoclonal protein was detectable by QIP-MS/EXENT (1:160 dilution) is shown in the top mass spectrum in (C). A much clearer residual monoclonal protein peak was observed when the sample was tested by FLC-MS (bottom mass spectrum in (C)). (D) shows the highest dilution at which residual lambda light chain monoclonal protein was detectable by FLC-MS (1:2560 dilution) at which point the FLC ratio was normal and there was no residual monoclonal protein detectable by QIP-MS/EXENT. All monoclonal protein peaks identified are highlighted with red mass labels.

Each specificity in the MALDI-TOF MS assay requires the development of a unique strategy for the EXENT software to permit the accurate identification and quantitation of monoclonal protein peaks due to the differing normal appearances within each specificity. This process requires the acquisition and evaluation of multiple rounds of training sets of data in collaboration with the software developers to allow refinement of the test strategy. Owing to the fact that the FLC-MS assay is still in the research and development phase, the software strategy for the automatic identification and quantitation of monoclonal proteins identified by FLC-MS has not yet been finalised. All FLC-MS spectra therefore have to be analysed manually in FlexAnalysis at present. Quantitative analysis of the monoclonal protein peaks identified by FLC-MS has therefore not been performed, so the sensitivity limit in mg/L of FLC-MS cannot be determined from this experiment.

The workflow for the preliminary FLC-MS assay is shown in Figure 3.5. Similar to the QIP-MS/EXENT assay sample processing and spotting is performed on the iP-500 liquid handler and samples are analysed on a Bruker microflex mass spectrometer. However the current version of the EXENT software cannot be used for automated interpretation of FLC-MS spectra.

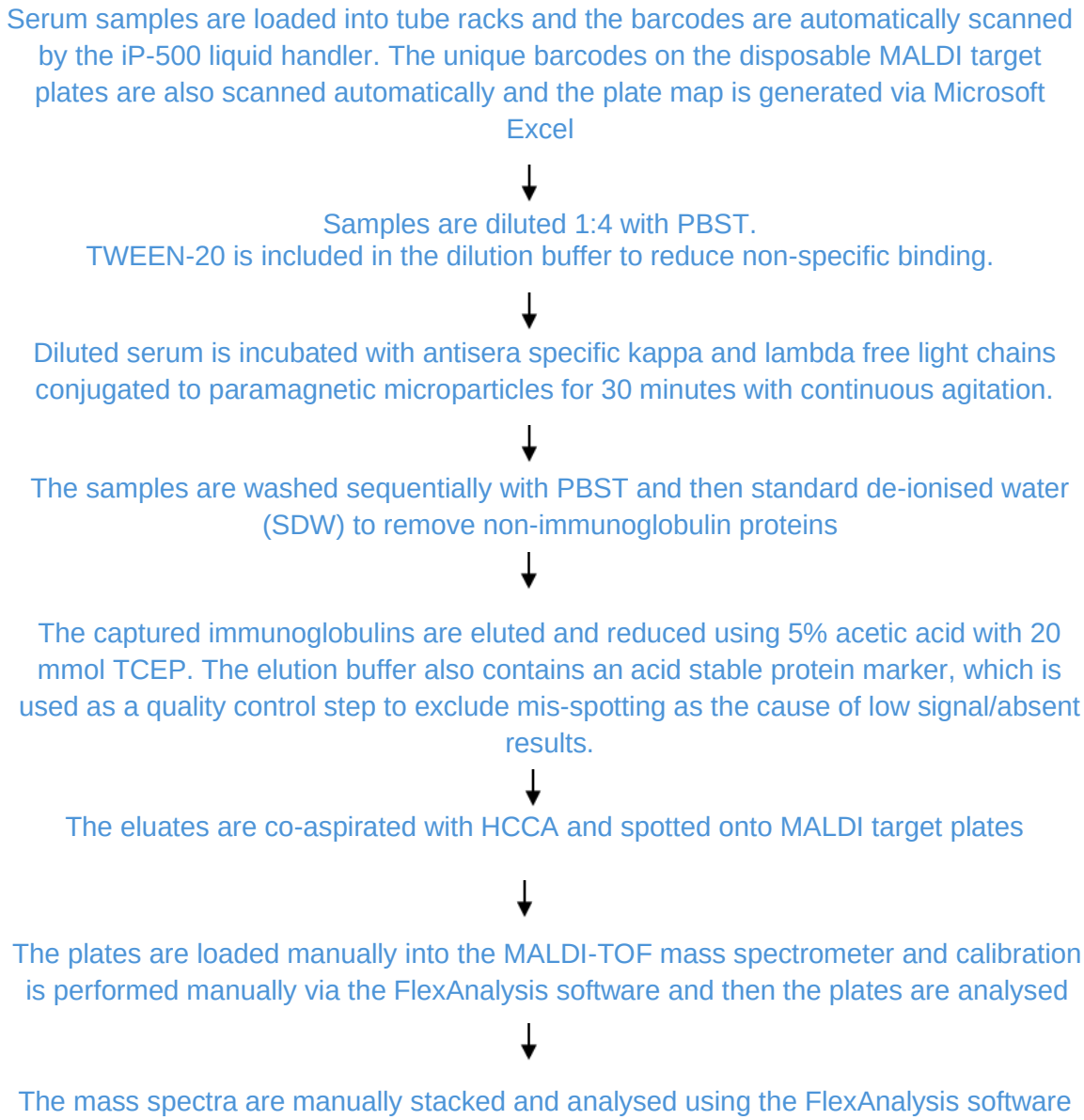


Figure 3. 5 Workflow for the prototype FLC-MS assay
Programmes for the liquid handler to perform the immune enrichment, elution and reduction and spotting onto MALDI target plates have been developed, including two plate scripts which have improved batch size from 40 to 72 samples per batch. Spectral analysis is performed manually in FlexAnalysis by trained operators as no automated software has yet been developed and validated for the interpretation of FLC-MS data.

3.2.3 Bead Chemistry Optimisation and Spotting Quality Control

During the early assay development work and early clinical studies, including IMAJEM and ALLG MM17, additional peaks at *m/z* 7800 were observed in all serum and plasma samples analysed (examples mass spectra shown in Figure 3.6). Further work undertaken by the mass spectrometry sub team led by Dr Simon North, determined

that these were due to single subunits of platelet factor 4 (PF4). An alternative second generation of paramagnetic bead chemistry, which has lower residual charge, was tested and eliminated this issue. Second generation chemistry beads were therefore used in the validation and verification of the QIP-MS/EXENT assay and for the clinical studies conducted as part of this thesis.

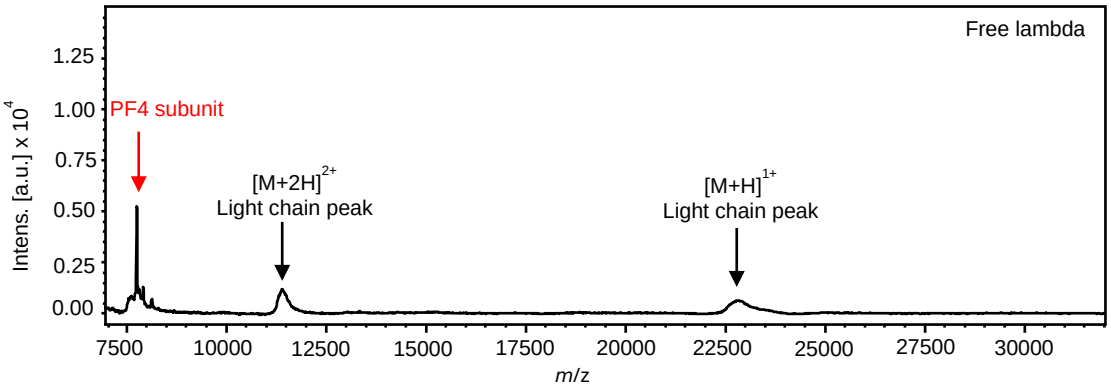


Figure 3. 6 PF4 Subunit Contamination in a Sample Analysed Using First Generation Bead Chemistry Reagents
In addition to the singly and doubly charged light chain peaks (highlighted by the black arrows) an additional peak is present at m/z 7800 (highlighted by the red arrow), with a distinct appearance compared to the polyclonal light chain peak. This peak is the result of a subunit of PF4 sticking to the beads due to low-level residual charge on the beads.

A low-cost acid stable protein with an *m/z* distinct from the mass regions for the singly, doubly and triply charged light chain regions was identified by the sub teams lead by Dr Simon North and Dr David Barnidge by screening panels of suitable candidate proteins. The protein identified is added to the elution buffer as a quality control check for the spotting stage of the assay. If a spotting error occurs, for example if little or no sample is aspirated due to the presence of an air bubble in the well, the error can be detected by the presence of a low intensity peak for the spotting control or an absent peak for the spotting control (Figure 3.7).

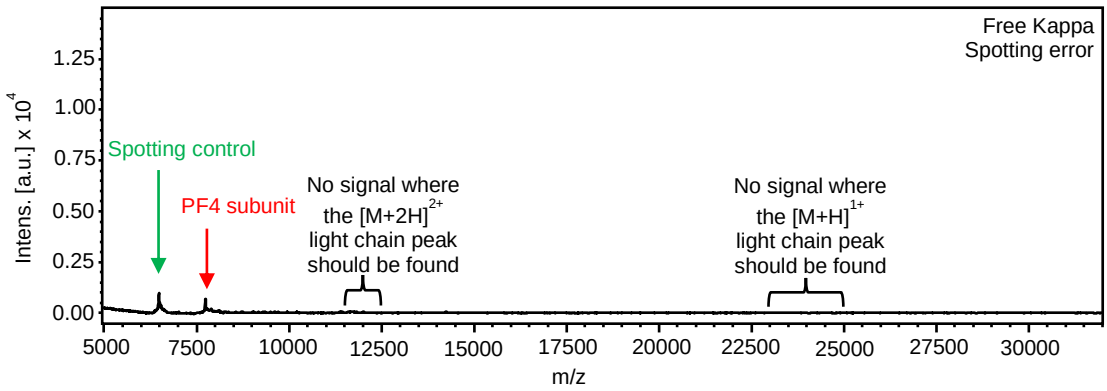


Figure 3. 7 Absent light chain signals due to mis-spotting is detected by examining the peak of intensity of the spotting control

No peaks are present in the m/z regions for the singly and doubly charged light chain ions (labelled with the black brackets) in this sample from the ALLG MM17 trial. The very low intensity peak for the spotting control (highlighted by the green arrow) confirms that the absence of light chain signal is due to an error in the spotting stage of the assay. The PF4 peak (red arrow) is also of low intensity due to error at the spotting stage.

3.2.4 Oligoclonal Peaks are Present in Most Post-induction Mass Spectra Obtained from Patients Treated in the ALLG MM17 Trial

To evaluate the suitability of using post-induction samples where no presentation sample was available the distribution of monoclonal protein peaks present in serial samples from patients treated in the ALLG17 study was evaluated. 31/50 (62.0%) patients from the trial had a baseline sample at least one follow-up sample with residual monoclonal protein detectable by MALDI-TOF MS. The median difference in the *m/z* of the light chain component of the monoclonal protein identified at baseline and follow-up was -2 (range -10 to +8) for the doubly charged light chain (Table 3.2). Peaks identified in follow-up spectra with a different isotype to the monoclonal protein identified and those with a difference between the *m/z* of the peak observed in follow-up spectra and baseline spectra was greater than 10 were labelled as oligoclonal peaks.

Table 3. 2 *m/z* distribution of monoclonal peaks identified at baseline and follow-up samples from patients treated in the ALLG17 trial who had detectable residual monoclonal protein in at least one of their follow-up samples

m/z of the monoclonal protein at baseline	*m/z* of the monoclonal protein pre-ASCT	*m/z* difference between baseline and pre-ASCT	*m/z* of the monoclonal protein post-ASCT	*m/z* difference between baseline and post-ASCT	*m/z* of the monoclonal protein post consolidation	*m/z* difference between baseline and post consolidation
11748	11746	2	Not tested*	n/a	Not tested*	n/a
11861	11863	-2	11863	-2	Not tested*	n/a
12007	12014	-7	12015	-8	Negative	n/a
11773	11772	1	Negative	n/a	Negative	n/a
12109	12119	-10	Negative	n/a	Negative	n/a
11533	11539	-6	Negative	n/a	Negative	n/a
12019	12021	-2	12021	-2	12017	2
11735	11741	-6	11738	-3	Negative	n/a
11589	Not tested*	n/a	11594	-5	Negative	n/a
11689	11690	-1	11690	-1	11689	0
11917	11916	1	Not tested*	n/a	Not tested*	n/a
11531	11532	-1	11533	-2	11533	-2
11589	Not tested*	n/a	Negative	n/a	11586	3
11982	11982	0	Negative	n/a	Not tested*	n/a
12155	12157	-2	Negative	n/a	Negative	n/a
12119	12117	2	12117	2	12120	-1

m/z of the monoclonal protein at baseline	*m/z* of the monoclonal protein pre-ASCT	*m/z* difference between baseline and pre-ASCT	*m/z* of the monoclonal protein post-ASCT	*m/z* difference between baseline and post-ASCT	*m/z* of the monoclonal protein post consolidation	*m/z* difference between baseline and post consolidation
11467	11471	-4	11469	-2	11465	2
11318	11318	0	11319	-1	11320	-2
11337	11344	-7	Not tested*	n/a	Not tested*	n/a
11716	11717	-1	11719	-3	11719	-3
11817	11814	3	11809	8	Not tested*	n/a
11603	11606	-3	11610	-7	11607	-4
11725	11726	-1	Not tested*	n/a	Not tested*	n/a
11537	11537	0	11544	-7	11544	-7
11781	11789	-8	Negative	n/a	Negative	n/a
11685	11687	-2	11677	8	11680	5
11749	11749	0	11746	3	Not tested*	n/a
11769	11776	-7	Negative	n/a	Negative	n/a
11642	11643	-1	Negative	n/a	Negative	n/a
11732	11727	5	Negative	n/a	Not tested*	n/a
11651	11653	-2	11653	-2	Not tested*	n/a

**These follow-up samples were not tested as there was no residual serum available for MS testing*

36 patients had paired MS results from baseline and the end of induction and in 22/36 (61.1%) samples from the end of induction at least one oligoclonal peak was detectable by MS (Figure 3.8A). 6/36 (16.7%) samples contained at least one oligoclonal peak with the same immunoglobulin isotype as the monoclonal protein identified in the baseline sample (Figure 3.8B). Example spectra showing the typical appearances of a monoclonal peaks in comparison to oligoclonal peaks and a polyclonal control sample are shown in Figures 3.8C-E.

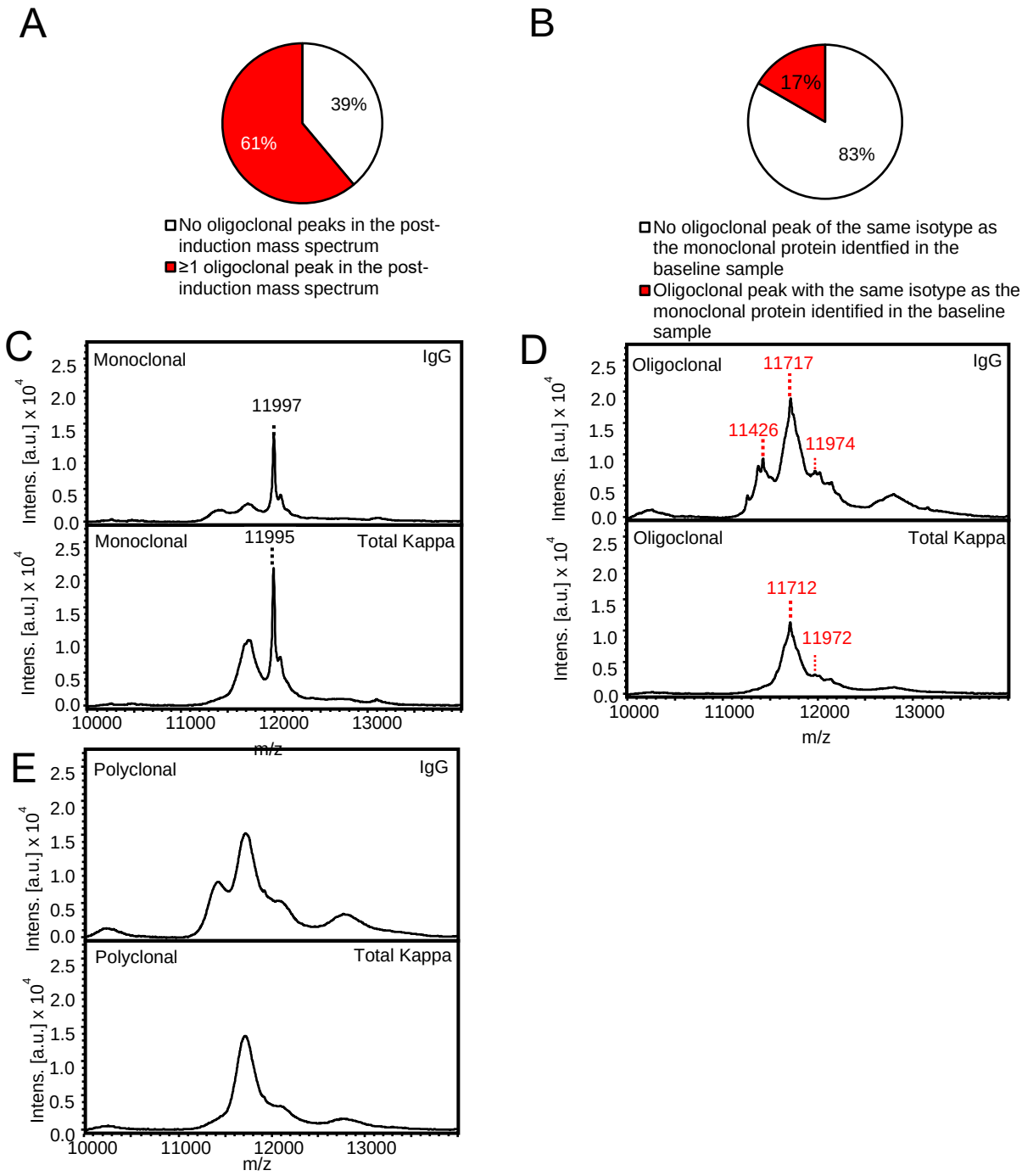


Figure 3. 8 Oligoclonal peaks were highly prevalent in mass spectra from samples taken at the end of induction chemotherapy in the ALLG17 trial

(A) shows the proportion of patients with ≥1 oligoclonal peak detectable by MALDI-TOF MS post-induction chemotherapy and (B) shows the proportion of patients with ≥1 oligoclonal peak with the same heavy chain and light isotype as the monoclonal protein identified in the baseline sample. (C-D) show the baseline (C) and post-induction (D) mass spectra from a patient with an oligoclonal peak of the same isotype as the monoclonal protein identified in the baseline sample. The IgG kappa monoclonal protein identified at baseline is highlighted by the black mass labels at m/z 11997 for the doubly charged light chains in (C). The IgG kappa oligoclonal peaks identified in the post-induction sample are highlighted by the red mass labels in (D) and they all have an m/z which is >10 different than the m/z of the monoclonal protein identified at baseline. Mass spectra from a polyclonal control sample are shown in (E).

In samples in which one or more oligoclonal peaks with the same isotype as the monoclonal protein identified in the baseline sample were identified in the post-induction sample, the median difference in the *m/z* between the monoclonal protein identified in the baseline sample and the oligoclonal peak observed in the post-induction was 82.5 (range -178-407) for the doubly charged light chains (Table 3.3).

Table 3. 3 *m/z* difference between monoclonal peaks identified at baseline and peaks of the same isotype labelled as oligoclonal peaks in mass spectra from post-induction samples

***m/z* of the monoclonal protein identified at baseline**	***m/z* of the oligoclonal peak identified post-induction of the same isotype as the MP**	***m/z* difference between the monoclonal protein and the oligoclonal peak**
12109	11702	407
11533	11711	-178
11997	11717	280
11568	11618	-50
11642	11757	-115
11337	11476	-139

These results suggest that post-induction samples will be unreliable to identify the *m/z* monoclonal protein, even if the isotype is known from prior electrophoretic analyses, in patients who have achieved a deep serological response by the end of induction. By utilising such a strategy there is risk that low-level oligoclonal peaks will be mis-identified as low-level residual disease and then the incorrect *m/z* would then be used to track the monoclonal protein produced by the neoplastic plasma cell clone through the subsequent samples.

3.2.5 Haemolysis does not interfere with the interpretation of mass spectra obtained following QIP-MS/EXENT testing

To assess whether haemolysis has a significant impact on the assessment of spectra obtained following QIP-MS/EXENT testing, serum samples from three healthy donors were obtained and the serum was separated on the day of sample collection, 24 hours post sample collection and seven days post sample collection and then tested using the QIP-MS/EXENT assay. Polyclonal immunoglobulin peaks were observed with no additional interfering peaks in 9/9 (100%) samples. Example mass spectra from the IgG eluates obtained from all three time points from one of the healthy donors are shown as overlaid spectra in Figure 3.9.

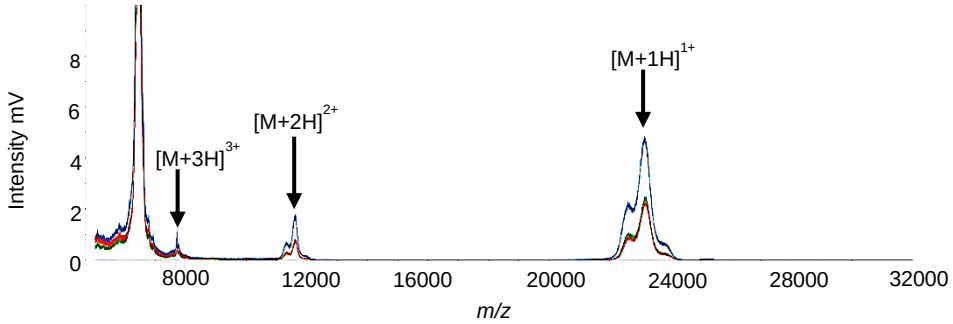


Figure 3. 9 Interpretable mass spectra can be obtained with QIP-MS/EXENT testing even after delayed sample separation

Mass spectra obtained from the IgG eluates tested by QIP-MS/EXENT following separation of serum on the day of sample collection (red mass spectrum), 24 hours post sample collection (green mass spectrum) and seven days post sample collection (blue mass spectrum). The peaks representing the singly, doubly and triply charged polyclonal light chains are highlighted by the black arrows.

3.2.6 High level monoclonal proteins cause antibody independent binding (AIB) in a small proportion a small proportion of patients which can be overcome by using a higher sample dilution factor

In 12/568 (2.1%) and 15/343 (4.4%) of samples analysed from the IMAJEM and GEM-CESAR trials peaks suggestive of monoclonal protein were observed in more than one specificity but with the same *m/z*. In most cases there was a dominant peak in one heavy chain specificity and one light chain specificity and much smaller peaks in the other three specificities. However, in a small number of samples peaks of similar

intensity were observed in all five spectra. This phenomenon was hypothesised to be due to high level monoclonal proteins as it predominantly occurred in baseline samples and was referred to as AIB.

The potential utility of using a higher sample dilution factor (1:50 rather than 1:10) to overcome AIB was explored in affected samples from the Myeloma XI trial and in all affected samples this resolved the AIB. Example mass spectra from a baseline sample from a patient treated in the Myeloma XI trial showing AIB when tested with a 1:10 dilution are shown in Figure 3.10A and the mass spectra obtained following testing of a sample diluted 1:50 with PBST are shown in Figure 3.10B. Following the 1:50 dilution the isotype of the monoclonal protein (IgG lambda in this example) can clearly be identified

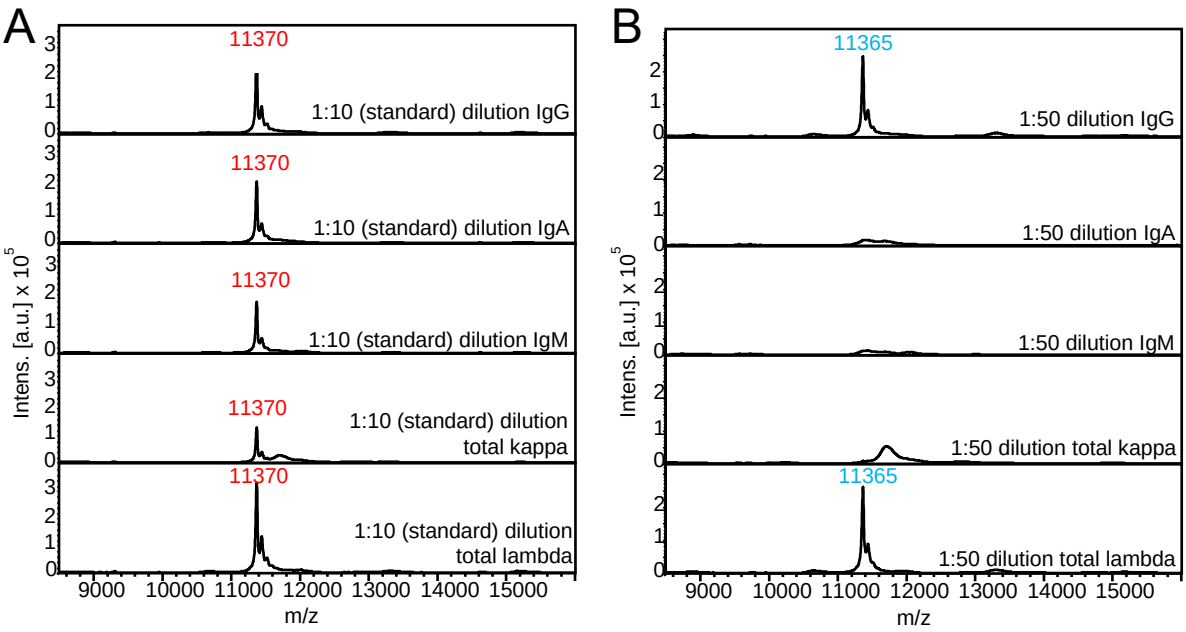


Figure 3. 10 AIB Occurs in A Small Proportion of Samples with High Level Monoclonal Proteins and Can be Overcome by Using a Higher Sample Dilution
(A) Shows an example mass spectrum for a sample with a large IgG lambda monoclonal protein in which significant AIB was observed when the standard sample dilution of 1:10 with PBST was used. The total IgG concentration in this sample was 76.23 g/L. In all five specificities a monoclonal peak at m/z 11370 is visible (highlighted by the red mass labels). The intensity of the peaks seen in the IgG, IgA and IgM spectra is similar, making identification of the monoclonal protein isotype difficult. (B) shows the mass spectra obtained for the same sample after QIP-MS/EXENT testing was performed following a 1:50 dilution in PBST. The IgG lambda monoclonal protein is highlighted by the blue mass labels.

For samples with less severe AIB in which the isotype of the monoclonal protein could easily be identified but minor peaks were visible in the uninvolved spectra, training data sets were sent to the software developers so that they could train the EXENT applet to automatically identify AIB and disregard any AIB peaks (Figure 3.11).

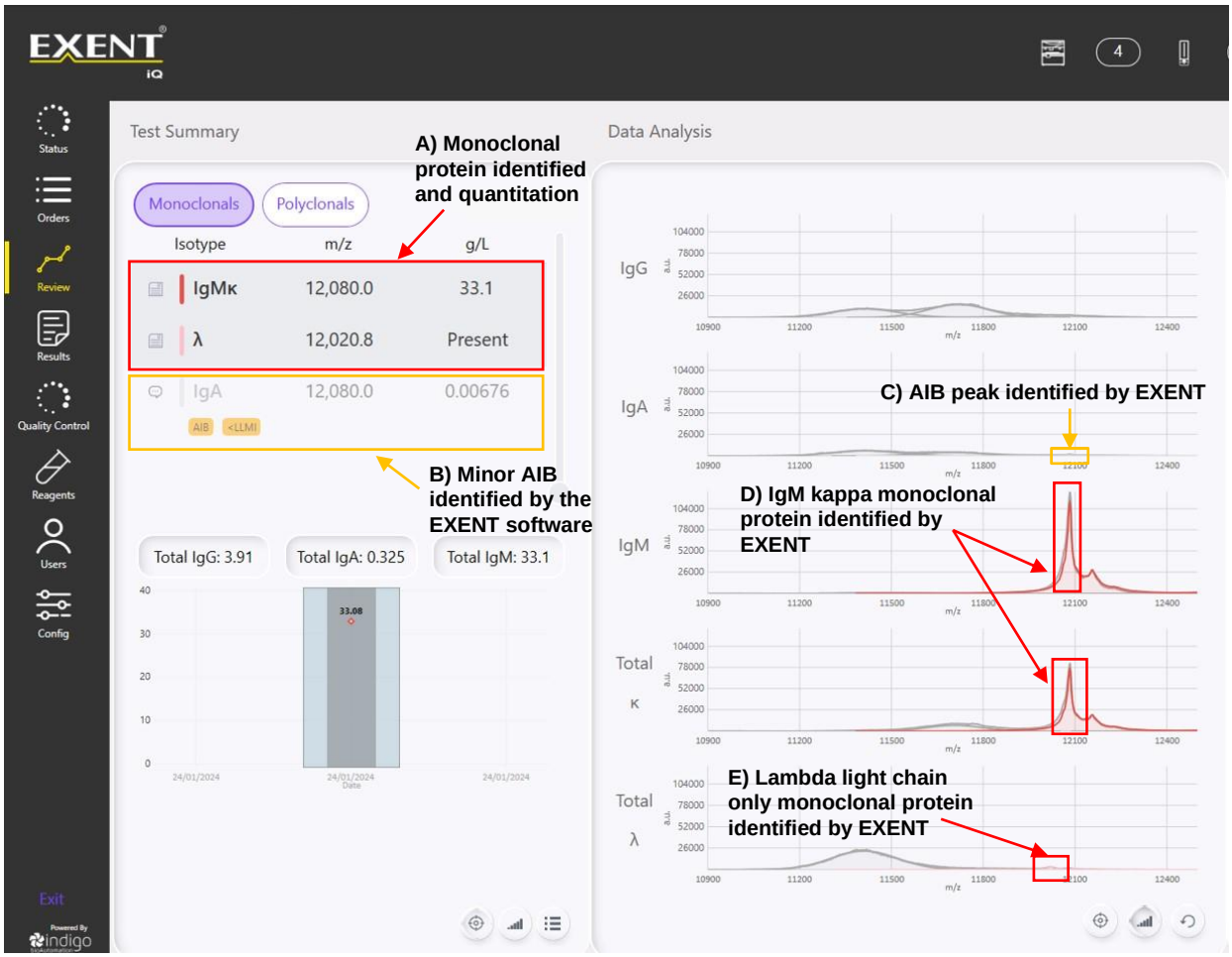


Figure 3. 11 AIB is recognised automatically by the EXENT software and excluded from the list of monoclonal proteins reported by EXENT

(A) Shows the monoclonal proteins identified by EXENT. The peaks representing the IgM kappa and lambda light chain only monoclonal proteins are highlighted by the red boxes and annotated with the labels (D) and (E) respectively. (B) Shows a very small AIB peak that was identified in the IgA mass spectrum that the software has disregarded as it has the same m/z as the IgM kappa monoclonal protein and a very low intensity consistent with minor AIB. The AIB peak is highlighted by the yellow box (C).

AIB was also frequently observed when the 1:2 sample dilution used in the early FLC-MS work in AL amyloidosis samples was applied to presentation samples from patients with multiple myeloma. This was overcome by using a 1:4 sample dilution for the FLC-MS assay, so all multiple myeloma samples that underwent FLC-MS testing in the clinical studies included in this thesis were tested using a 1:4 dilution with PBST.

3.2.7 IVD Regulation Certification for the QIP-MS Assay and Assay Performance

The QIP-MS/EXENT assay obtained IVD regulation certification in August 2023. The workflow for the approved assay is shown in Figure 3.12. The assay is performed using two analysers – an EXENTiP analyser for the immune enrichment and spotting steps and a Bruker microflex mass spectrometer for the MS analysis. The software associated with the assay is called the EXENT software and this software automatically interprets the mass spectra. The assay has a sensitivity of 15mg/L for IgG, IgA and IgM monoclonal proteins .

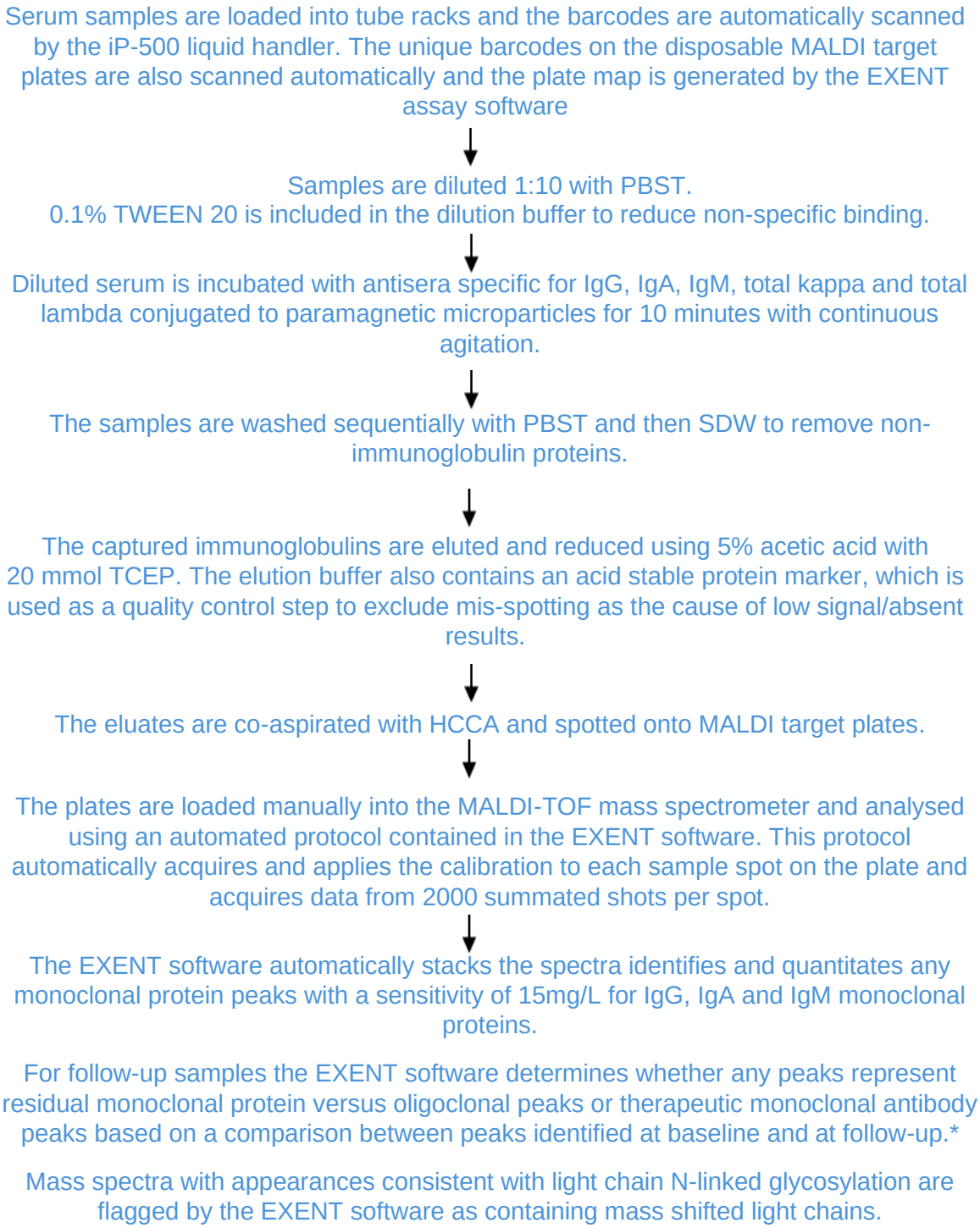


Figure 3. 12 Workflow and performance characteristics of the EXENT assay
The EXENT assay automatically processes serum samples utilising reagents specific for IgG, IgA, IgM, total kappa and total lambda. The spectra are automatically stacked and interpreted by the EXENT software. The assay is able to quantitate IgG, IgA and IgM monoclonal proteins down to 15mg/L and automatically flags samples that contain light chains with N-linked glycosylation.

**using standard calibration a monoclonal protein peak in a follow-up sample within m/z +/-10 of the peak identified for the doubly charged light chain peak in the baseline sample is consistent with residual disease. The m/z variation between samples can be reduced to m/z +/- 4 when the spotting control is also used as a "lock mass" to provide an internal mass standard within each sample spot. The additional mass calibration using the lock mass is automatically applied by the final version EXENT software.*

3.2.8 Development of a Reflex Test to Confirm the Presence of Light Chain N-linked Glycosylation

N-linked light chain glycosylation results in broader polytypic peaks with a higher *m/z* than non-glycosylated monoclonal light chains. The presence of multiple peaks is due to the addition of variable numbers of carbohydrate molecules, each of which is represented by one of the peaks within hedgehog shaped peak area. The previous sensitivity comparison between MS and Freelite suggested that the heterogeneous nature of these peaks may impact sensitivity. Therefore work was undertaken to identify a methodology for de-glycosylation using peptide: N-glycosidase F (PNGase F), which is an enzyme that removes N-linked oligosaccharides, that could be coupled to QIP-MS/EXENT and/or FLC-MS.

3.2.8.1 Development of a Protocol For De-glycosylation using PNGase F that could be Combined with the QIP-MS/EXENT Assay

PNGase F is active in the pH range 6-10 (Promega, 2024) but the 5% acetic acid used in the elution buffer has a pH of 2.4, so neutralization using triethylammonium bicarbonate (TEAB) prior to the addition of PNGase F was evaluated. However, this yielded very low-intensity mass spectra likely as a result of salt creation, which induced suppression of the light chain ion signals (Figure 3.13).

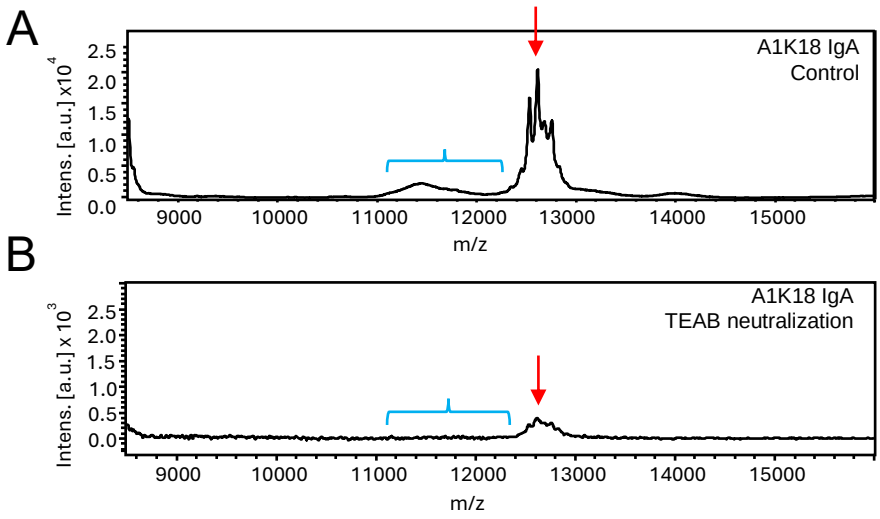


Figure 3. 13 Neutralisation with TEAB causes ion suppression resulting in low intensity mass spectra

The mass spectrum obtained following standard QIP-MS/EXENT testing of the myeloma library sample A1K18 is shown in (A). (B) shows the mass spectra obtained when the sample was neutralised with TEAB following the elution and reduction step. The glycosylated light chains (highlighted by the red arrows) are visible with very low intensity in the mass spectrum obtained from the sample neutralised with TEAB (B) compared to the glycosylated light chains seen in the control sample (A) and no polyclonal light chain signal is visible in the neutralised sample (the m/z area for the doubly charged polyclonal light chains is highlighted with the blue brackets in each mass spectrum).

On-bead de-glycosylation prior to elution was therefore explored. No de-glycosylation was observed after incubation with 1µL PNGase F for five hours, however partial de-glycosylation was achieved following five hour incubation with 2µL PNGase F (Figure 3.14).

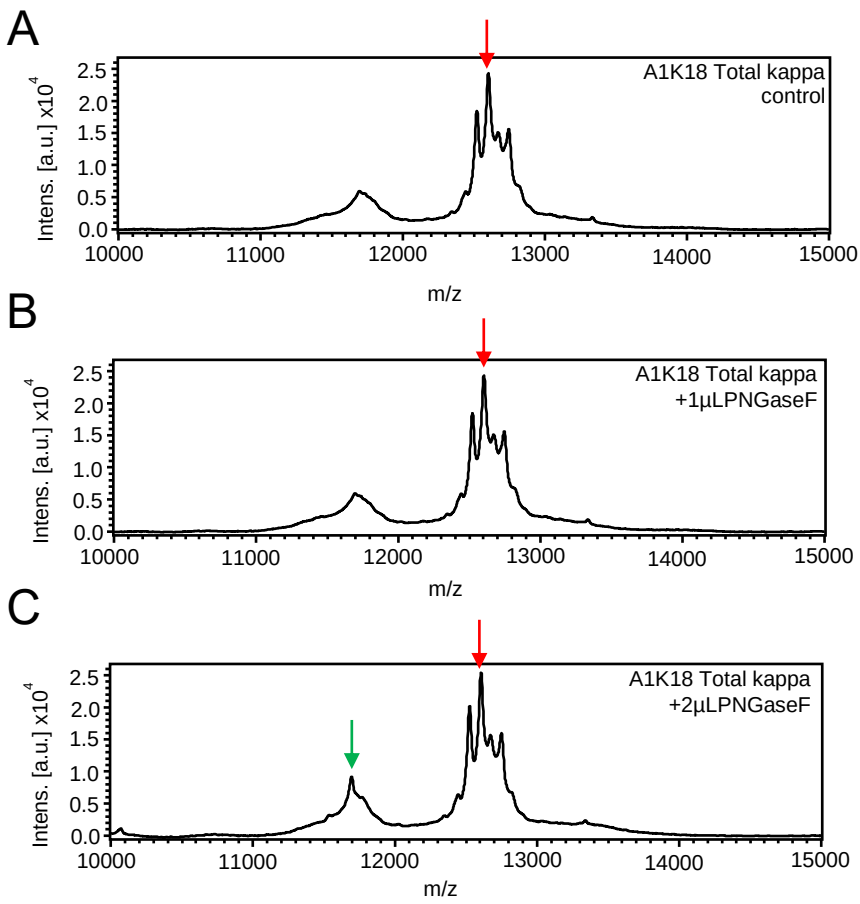


Figure 3. 14 On-bead de-glycosylation of captured immunoglobulins prior to elution and reduction

(A) Shows the mass spectrum for A1K18 tested using the standard QIP-MS/EXENT assay. The mass spectra obtained following five hours incubation with 1µL and 2µL PNGase F prior to elution and reduction are shown in (B) and (C) respectively. The polytypic peaks representing the doubly charged glycosylated light chains are highlighted by the red arrow in each spectrum. Following incubation with 2µL PNGase F a small monoclonal peak without light chain glycosylation is visible, which is highlighted by the green arrow in (C).

Owing to the fact that de-glycosylation of non-denatured proteins is less efficient (Szabo *et al*., 2010), a time course of incubation times with PNGase F was conducted to determine if longer incubation with PNGase F could yield a greater degree of de-glycosylation. This was undertaken rather than exploring further increasing the volume of PNGase F used per analysis due to the relatively high cost of this reagent (50µL of PNGase from Promega UK costs £182 in 2024) (Promega, 2024).

A significant increase in the amount of de-glycosylation achieved was observed between 24 hours incubation with PNGase F and 72 hours incubation with PNGase F (Figure 3.15). This is represented by an increase in the size of the non-glycosylated monoclonal protein peak and a reduction in the peak area representing the residual glycosylated monoclonal protein peak.

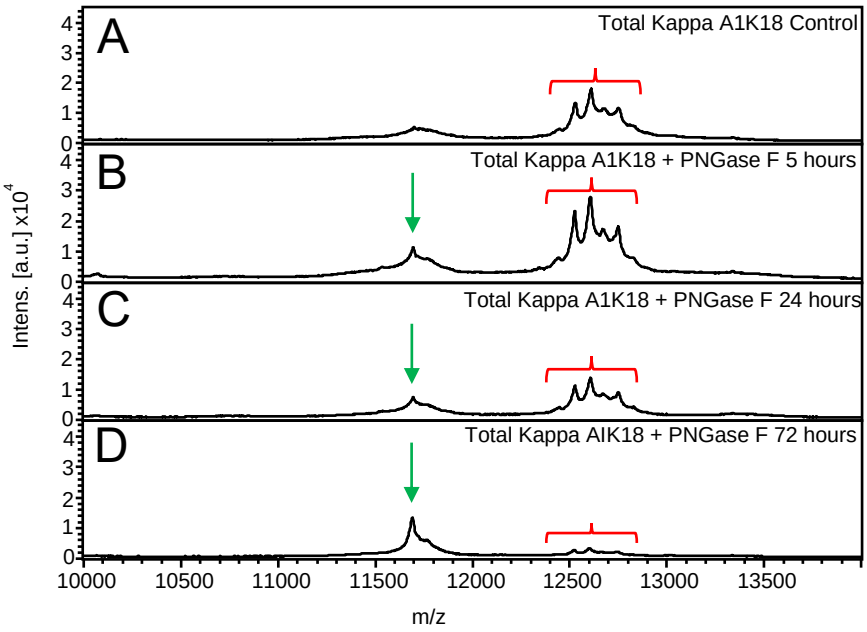


Figure 3. 15 Time course of on-bead de-glycosylation using PNGase F
(A) shows the control mass spectrum obtained from A1K18. The amount of de-glycosylation achieved after incubation with 2µL PNGase F for 5, hours, 24 hours and 72 hours are shown in (B),(C) and (D) respectively. The doubly charged glycosylated light chains are highlighted by the red brackets in each mass spectrum and the de-glycosylated monoclonal light chain peak is highlighted by the green arrows in (B-D).

Additional peaks consistent with PNGase F were observed in the *m/z* region for doubly charged light chains when PNGase F supplied by Promega rather than Roche was used for on-bead de-glycosylation and these could not be eliminated by post incubation washes with water or PBST (Figure 3.16). Comparison between the storage buffers that the PNGase F supplied in by Roche and Promega revealed that the concentration of EDTA differed between the two (12.5 mM versus 5 mM EDTA)

and performing an additional wash with 50mM EDTA prior to incubation with PNGase F eliminated the PNGase F contamination.

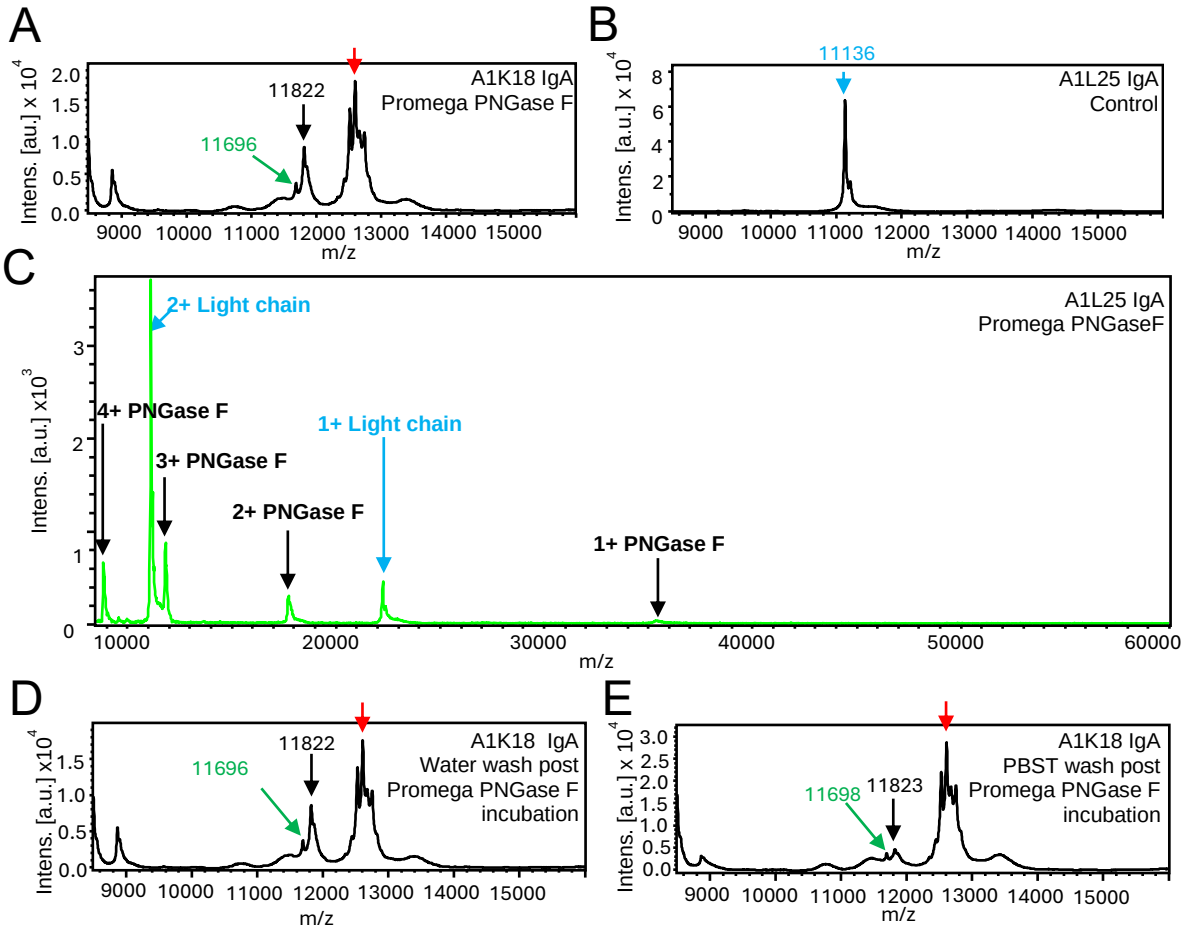


Figure 3. 16 PNGase F Contamination following On-bead De-glycosylation with PNGase F Supplied by Promega

(A) Mass spectra obtained using PNGase F supplied by Promega contained an additional peak at m/z 11822 (highlighted by the black arrow). This was unrelated to the doubly charged de-glycosylated light chains (highlighted by the green arrow) and the residual glycosylated monoclonal light chains (highlighted by the red arrow). (B) The same peak was observed at m/z 11822 in A1L25, which is a sample that contains an IgA lambda monoclonal protein from the myeloma library. Analysis of the charge states for this protein over a wider mass range showed that the peak at m/z 11822 was consistent with the triply charged ions of PNGase F. The peaks representing the singly, doubly, triply and quadruply charge ions of PNGase F are highlighted by the black arrows in (C). The singly and doubly charged ions of the monoclonal protein peak from A1L25 are highlighted by the blue arrows in (C). (D) Shows that an additional water wash did not reduce the size of the Promega PNGase F peak (highlighted by the black arrow). (E) An additional wash with PBST after incubation with Promega PNGase F reduced the size of the PNGase F peak (highlighted by the black arrow) but did not completely eliminate it. The peak for the doubly charged light chains of the de-glycosylated monoclonal protein are highlighted by the green arrows in (D) and (E) and the residual non-glycosylated monoclonal protein is highlighted the red arrows in (D) and (E).

3.2.8.2 Comparison Between the Degree of De-glycosylation Achieved with On-bead De-glycosylation Coupled with FLC-MS Compared to QIP-MS/EXENT

Due to the fact that heavy chains are also glycosylated it was hypothesised that on-bead glycosylation using FLC specific beads may be more efficient. To assess this the degree of de-glycosylation achieved after on-bead de-glycosylation of the library sample A1K18 using IgA, total kappa and free kappa specific reagents was compared. This testing confirmed that highest rate of de-glycosylation was observed following on-bead de-glycosylation using free kappa specific reagents (Figure 3.17).

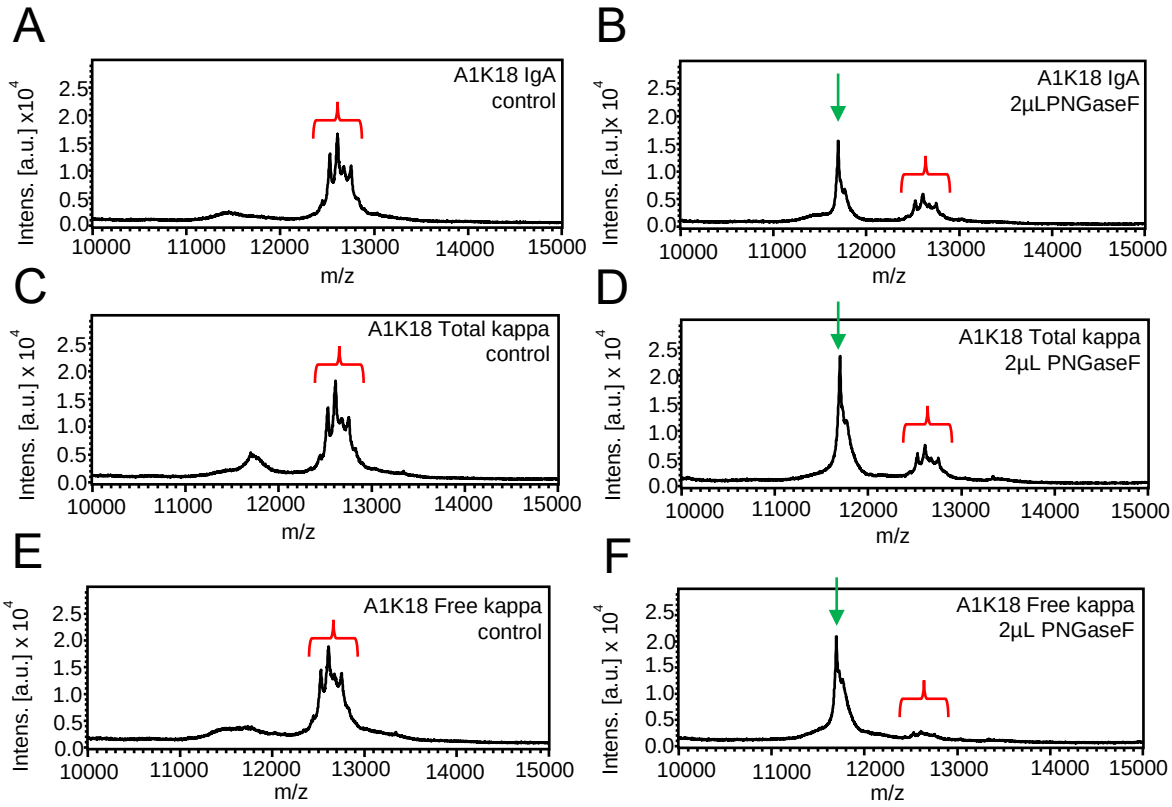


Figure 3. 17 Comparison of the degree of glycosylation achieved by on-bead de-glycosylation with PNGase F using IgA and total kappa specific beads versus FLC specific beads

The spectra for A1K18 obtained after QIP-MS/EXENT and FLC-MS analysis without treatment with PNGase F are shown in (A), (C) and (E). Large polytypic peaks representing glycosylated light chains are visible in each mass spectrum and are highlighted by the red brackets. The degree of de-glycosylation achieved for IgA, total kappa and free kappa beads following incubation with PNGase F for 72 hours are shown in (B), (D) and (F). The de-glycosylated monoclonal peak is highlighted by the green arrows in (B), (D) and (F) and the residual glycosylated light chain peaks are highlighted by the red brackets.

3.2.8.3 Comparison Between the Standard FLC-MS Assay, FLC-MS Coupled with On-bead De-glycosylation and Serum FLC Assessments for Residual Disease Detection in Five Patients from the ALchemy Study

To assess whether de-glycosylation could enhance the sensitivity of FLC-MS for the detection of residual monoclonal FLC, five samples from patients in the ALchemy study who had glycosylated monoclonal FLC at baseline and achieved FLC-MS negativity at six and/or 12 months follow-up were tested with FLC-MS coupled with de-glycosylation. The results of this testing are summarised in Table 3.4.

Table 3. 4 Detection of residual monoclonal FLC Using FLC-MS Coupled with on-bead de-glycosylation compared to standard techniques and FLC-MS without on-bead de-glycosylation in samples from five patients in the ALchemy study

Sample ID	SPE (g/L)	IFE	iFLC (mg/L)	serum FLC ratio	BJP	FLC-MS	FLC-MS coupled with on-bead de-glycosylation (m/z)
221 baseline	Negative	Lambda light chains	311.4	0.03	Lambda	Glycosylated lambda FLC	Lambda FLC (11421)
221 6 months	Negative	Negative	11.8	0.97	Negative	Negative	Lambda FLC (11419)
221 12 months	Negative	Negative	13.7	0.77	Negative	Glycosylated lambda FLC	Lambda FLC (11419)
604 baseline	Negative	Negative	82.7	3.27	Negative	Glycosylated kappa FLC	Kappa FLC (11711)
604 6 months	Negative	Negative	88.6	2.09	Not available	Negative	Kappa FLC (11709)
604 12 months	Negative	Negative	106.3	2.29	Negative	Negative	Negative
573 baseline	Negative	Negative	336	18.6	Kappa	Glycosylation kappa FLC	Kappa FLC (12067)
573 6 months	Negative	Negative	328.2	9.71	Kappa	Negative	Kappa FLC (12066)
573 12 months	Negative	Negative	197.5	5.4	Not available	Negative	Kappa FLC (12066)
182 baseline	6	IgG lambda	156	0.16	Lambda	Glycosylated lambda FLC	Lambda FLC (11347)
182 6 months	No sample	No sample	No sample	No sample	No sample	No sample	No sample
182 12 months	Negative	Negative	23.8	1.22	Negative	Negative	Lambda FLC (11349)
242 baseline	Negative	Negative	563.5	39.1	Kappa	Glycosylated kappa FLC	Kappa FLC (11768)
242 6 months	Negative	Negative	25.2	1.5	Negative	Negative	Negative
242 12 months	Negative	Negative	26.5	1.28	Negative	Negative	Negative

4/5 (80.0%) patients had a follow-up samples from six months available for testing and in 3/4 (75.0%) samples residual monoclonal FLC was detectable by MS following on-bead de-glycosylation. Residual disease was detectable using the electrophoretic assays and serum FLC assessments in 1/4 (25.0%) of these samples.

All patients had follow-up samples from 12 months available for FLC-MS coupled with on-bead de-glycosylation testing; 4/5 patients were negative for residual monoclonal FLC using the standard FLC-MS assay and 1/5 (20.0%) who had previously been negative by FLC-MS at six months had had re-emergence of detectable monoclonal FLC by FLC-MS at 12 months. Residual disease was detectable using on-bead de-glycosylation coupled with FLC-MS in 3/5 (60.0%) of these samples (example mass spectra are shown in Figure 3.18).

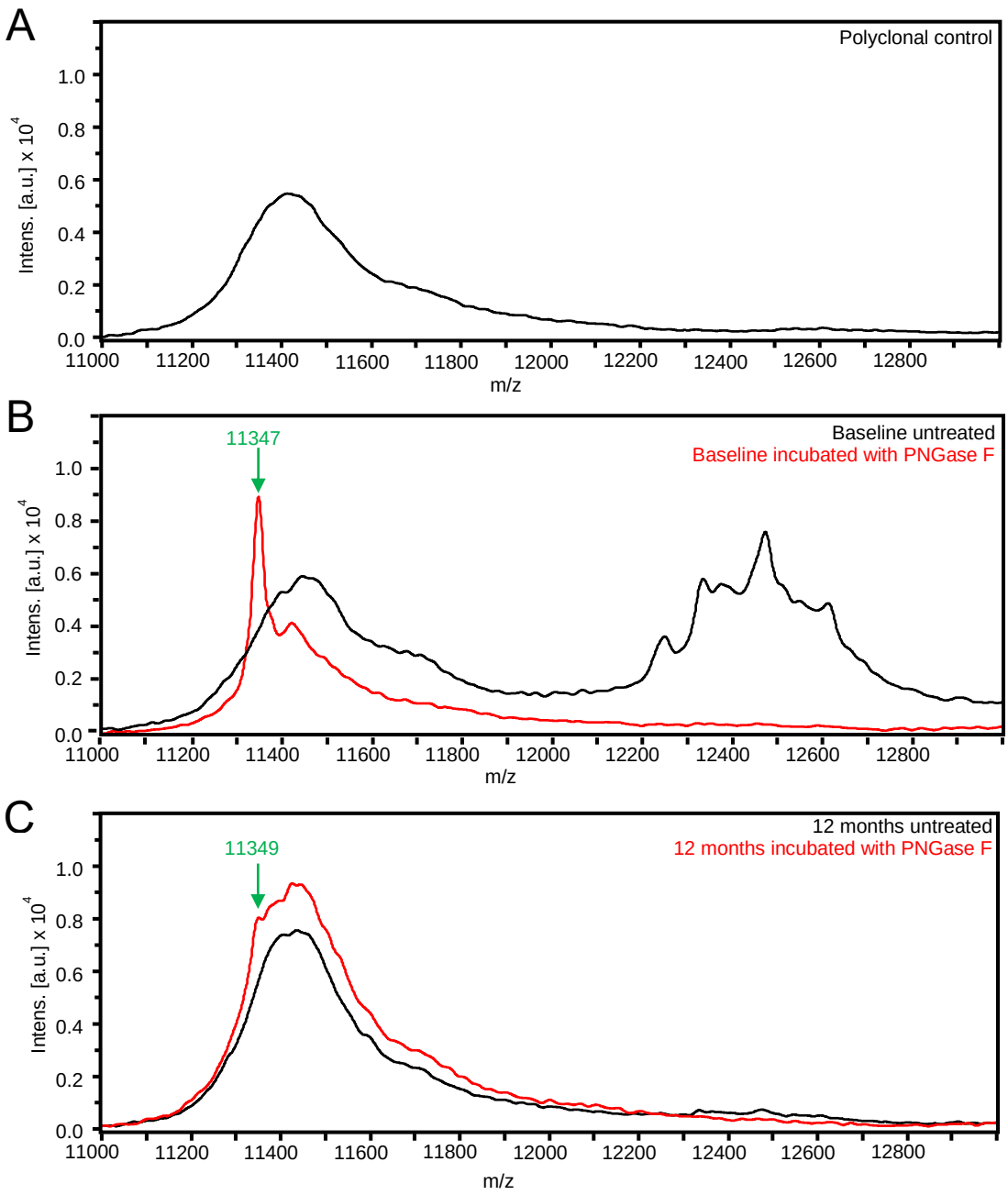


Figure 3. 18 On-bead de-glycosylation using PNGase F can enhance the sensivity of FLC-MS for the detection of residual monoclonal FLC in patients with N-linked glycosylation.

Overlays of the mass spectra obtained following standard FLC-MS testing and FLC-MS coupled with on-bead de-glycosylation for paired samples from baseline and six months follow-up are shown in (B) and (C). The spectra obtained following standard FLC-MS testing are shown in black and the spectra obtained after FLC-MS coupled with on-bead de-glycosylation are shown in red. The mass spectrum obtained for the free lambda eluate from a polyclonal control sample is shown in (A). Following on-bead de-glycosylation monoclonal lambda FLC were detectable by MS at m/z 11347 for the doubly charged light chains (highlighted by the green arrow in (B)). At 12 months no residual monoclonal FLC were detectable using the standard FC-MS but low-level monoclonal lambda FLC were detectable by MS at m/z 11349 for the doubly charged light chain following on-bead de-glycosylation (highlighted by the green arrow in (C)).

3.3 Discussion

This work has demonstrated that testing urine in addition to serum using the standard QIP-MS/EXENT assay protocol would not provide additional sensitivity for the detection of low-level monoclonal FLC. Urine samples were therefore not included in the clinical studies described in chapters four to seven.

In keeping with the variable sensitivity of the five bead MASS-FIX assay for the detection of low-level monoclonal FLC previously reported in comparison to the standard techniques (Dispenzieri *et al*., 2020a, Wilhite *et al*., 2021, Mellors *et al*., 2021), variable sensitivity of QIP-MS/EXENT in comparison to Freelite was observed. This work has shown that this is likely due to several factors including the proximity of the monoclonal light chain peak to the apex of the polyclonal background peak and the presence or absence of light chain N-linked glycosylation. Utilising FLC-MS provides additional sensitivity as it removes a significant proportion of the polyclonal background that comes from the light chains that have been released from the intact immunoglobulin background following the elution and reduction step of the QIP-MS/EXENT assay. The most sensitive panel for an intact light chain MALDI-TOF MS assay to assess for low-level residual disease in patients with multiple myeloma would therefore combine QIP-MS/EXENT with FLC-MS.

As the assays do not include an internal standard for quantitation, indirect quantitation is performed utilising the immunoglobulin measurements obtained by nephelometry multiplied by the percentage area represented by the monoclonal peak. This methodology permits the quantitation of intact immunoglobulin monoclonal protein

peaks with a sensitivity of 15 mg/L (Sakrikar *et al*., 2021), however as there is no assay that specifically quantitates total kappa and total lambda light chains, so only qualitative results for FLC only monoclonal proteins can be obtained from QIP-MS/EXENT testing. Therefore no quantitative assessment of the sensitivity of the QIP-MS/EXENT assay for the detection of FLC monoclonal proteins could be undertaken. Utilising FLC-MS quantitation would be possible using the total concentration of kappa and lambda FLC obtained by Freelite® testing. However, the software to identify monoclonal FLC peaks in FLC-MS spectra and calculate the percentage area it represents as a proportion of the total FLC peak is still under development. Therefore it was not possible to evaluate in mg/L the sensitivity limit of the FLC-MS assay. When this work is undertaken in the future it would be important that samples from patients with renal failure, who will have a higher polyclonal background, are included to assess any impact of this on assay sensitivity.

The high rate of oligoclonal peaks observed in the spectra from the end of induction chemotherapy in samples from the ALLG17 trial, indicates that there would be a significant risk of identifying the incorrect peak as the monoclonal protein peak to track if baseline samples were substituted for samples from the end of induction chemotherapy. Therefore in the studies conducted in chapters four to seven only patients with residual serum available for MS testing within one cycle of starting treatment will be included.

On-bead de-glycosylation enhances the sensitivity of the FLC-MS assay for the detection of residual monoclonal FLC in patients with light chain N-linked

glycosylation. This assay could be used to confirm the presence of residual monoclonal FLC in samples with equivocal results. However, the high cost and long sample processing time mean that the protocol should be further refined if it is to be explored as an add-on test for routine clinical use.

4: THE UTILITY OF MASS SPECTROMETRY FOR THE DETECTION OF RESIDUAL DISEASE IN PATIENTS WITH NEWLY DIAGNOSED SYMPTOMATIC MULTIPLE MYELOMA

4.1 Introduction

MS assays are emerging as a new and sensitive serum-based methodology for monitoring patients with PCD (Mills *et al*., 2015, North S, 2018, Spencer A, 2019, Puig N, 2019, Sakrikar *et al*., 2021, Derman *et al*., 2021, Puig *et al*., 2022). However, the optimal technique and panel of antisera has not been established. In addition, there are limited data available on the prognostic implications of MS-assessed treatment responses in patients undergoing treatment for multiple myeloma, particularly amongst patients receiving maintenance therapy, which is now considered standard of care in many countries (Mikhael *et al*., 2019, Sive *et al*., 2021, Dimopoulos *et al*., 2021).

In a study of 61 patients who were in CR and MRD negative by NGF at day +100 (+/- 60 days) post ASCT, Abeykoon *et al* reported that patients who were MASS-FIX negativity at had improved PFS compared to patients who remained positive by MASS-FIX. Overall, residual monoclonal protein was detectable by MASS-FIX in 59%

of the patients included in this study. However, the rate of residual monoclonal protein detection was much higher in patients with IgG myeloma compared to those with IgA and FLC-only myeloma (87% of patients with IgG myeloma had residual disease versus 33% and 30% of patients with IgA and FLC-only myeloma respectively) (Abeykoon *et al*., 2021). This suggests that some of the patients with IgG myeloma with residual monoclonal protein detectable by MASS-FIX may have been false positives due to the long half-life of IgG owing to its recycling through the neonatal Fc receptor. It is also unclear whether the lack of inclusion of FLC-specific reagents contributed the lower rate of residual disease detected by MASS-FIX in patient with FLC only myeloma.

The retrospective analysis of samples from patients treated in the STAMINA trial using MASS-FIX by Dispenzieri *et al* is one of only two only large-scale MS-based studies conducted to date which has included patients receiving post-transplant maintenance (Dispenzieri *et al*., 2022). In this study, the samples used to identify the monoclonal protein isotype and *m/z* of the light chain component of the monoclonal protein were post-induction samples and 47% of samples came from patients in ≥VGPR. It is possible that small oligoclonal bands may have been misassigned as low-level residual monoclonal protein in some of these patients, resulting in incorrect assignment of the MS-based response categorisation. However, despite this limitation MASS-FIX positivity pre-ASCT, pre-maintenance and at one year post-maintenance was still prognostic for PFS on univariate analysis. On multivariate analysis both MRD and MASS-FIX status at one year post enrolment were prognostic for OS but neither IFE status nor CR were (Dispenzieri *et al*., 2022).

Similar to the MASS-FIX assay, the QIP-MS/EXENT assay uses antisera specific for IgG, IgA, IgM, total kappa and total lambda but it has a lower reported limit of detection of 15 mg/L for intact immunoglobulin monoclonal proteins (Mills *et al*., 2016, Ashby JA, 2018). EXENT has frequently been combined with FLC-MS, which uses antisera specific for free kappa and free lambda light chains, in clinical studies (Spencer A, 2019, Puig N, 2019, Puig *et al*., 2022, Derman *et al*., 2021). There are no published studies to date on the comparing the limit of detection of FLC-MS compared to assays only utilising antisera specific and only limited data from clinical studies on whether the FLC specific reagents provide additional sensitivity (Puig *et al*., 2022, Huhn *et al*., 2022).

In an analysis of the first 223 patients treated in the PETHEMA/GEM2012 MENOS65 trial, Puig *et al* reported that in patients who were negative by IFE residual monoclonal protein was detectable using QIP-MS/EXENT and FLC-MS in over 10% of patients at the end of induction chemotherapy, post-ASCT and post-consolidation. Patients with residual positivity by MALDI-TOF MS had shorter PFS compared to MS-negative patients. In this study FLC-MS only provided additional sensitivity in 5% of patients (45.5% of these patients were classified as having FLC only myeloma using IFE and 54.5% had intact immunoglobulin secreting disease) (Puig *et al*., 2022). However, Huhn *et al* reported that FLC-MS provided additional sensitivity compared to QIP-MS/EXENT alone in a greater proportion of patients in CR (residual monoclonal protein was only detectable by FLC-MS in 28% and 25% of patients in CR post induction and post consolidation, respectively) (Huhn *et al*., 2022).

The second MS-based study which has included patients who received post-ASCT maintenance, was a study which included samples from 480 patients from the GMMG-MM5. This study utilised the QIP-MS/EXENT and FLC-MS assays and reported that MS negativity at all time points was associated with improved PFS (Mai *et al*., 2023). Although this trial included patients who were randomised to receive maintenance versus observation, the induction treatment used was old fashioned as it did not combine a proteasome inhibitor with an immunomodulatory drug, which is currently standard first-line treatment for transplant-eligible patients with multiple myeloma (plus an-anti-CD38 monoclonal antibody where funding is available).

MRD studies using approved methodologies have highlighted the prognostic benefit of sustained MRD negativity and this is being explored as a potential guide for the appropriateness of maintenance therapy cessation (Krishnan et al., 2020, Costa et al., 2021b, Royle et al., 2022, Derman et al., 2022). The prognostic utility of sustained negativity by MALDI-TOF MS has not been evaluated in any of the clinical studies that have been published to date.

The aims of the work included in this chapter were to:

- Compare the rates of residual disease detected by MS to a) the rates of residual disease detection in the serum using electrophoretic techniques and serum FLC assays and b) the rate of MRD positivity in the bone marrow by flow cytometry in patients with newly diagnosed multiple myeloma treated in the KCRD arm of the Myeloma XI trial

- Compare the rates of residual monoclonal FLC detection by Freelite® compared to FLC-MS in patients who become IFE negative following anti-myeloma treatment in the KCRD arm of the Myeloma XI trial
- Compare the prognostic significance of IFE, MS, and bone marrow based MRD assessments in patients undergoing treatment with KCRD in the Myeloma XI trial

4.2. Methods

Owing to the higher reported overall response rate and superior MRD results reported for the patients treated in the KCRD arm of the Myeloma XI trial (Jackson *et al*., 2021), only samples from patients who received KCRD were included in this study. All patients from this arm of the Myeloma XI trial who had sufficient serum from baseline and/or post cycle one and at least one response assessment sample from between the end of induction chemotherapy and day+100 post ASCT for MS testing were included in this study.

MALDI-TOF MS testing using the QIP-MS/EXENT and FLC-MS assays was performed on all samples. Quantitative analysis of the QIP-MS/EXENT results was performed retrospectively once the protype software for analysing the mass spectra had been developed. This was performed using the total immunoglobulin results from the central laboratory analysis. FLC-MS spectra were analysed manually and interpreted as positive if a peak of the same FLC isotype and with an *m/z* within +/-10 of the doubly charged light chain of the monoclonal FLC identified at baseline was present in the follow-up mass spectrum.

4.3.Results

4.3.1 Characteristics of the Patients from the Myeloma XI+ Trial Included in this Study

526 patients were randomised to receive KCRD followed by a high-dose melphalan conditioned ASCT. 293/526 patients had residual serum available for MALDI-TOF MS testing from presentation or post cycle one of induction chemotherapy and least one follow-up sample between the end of induction chemotherapy and day+100 post-ASCT. The time points and numbers of samples from each time point included in this study are shown in Figure 4.1.

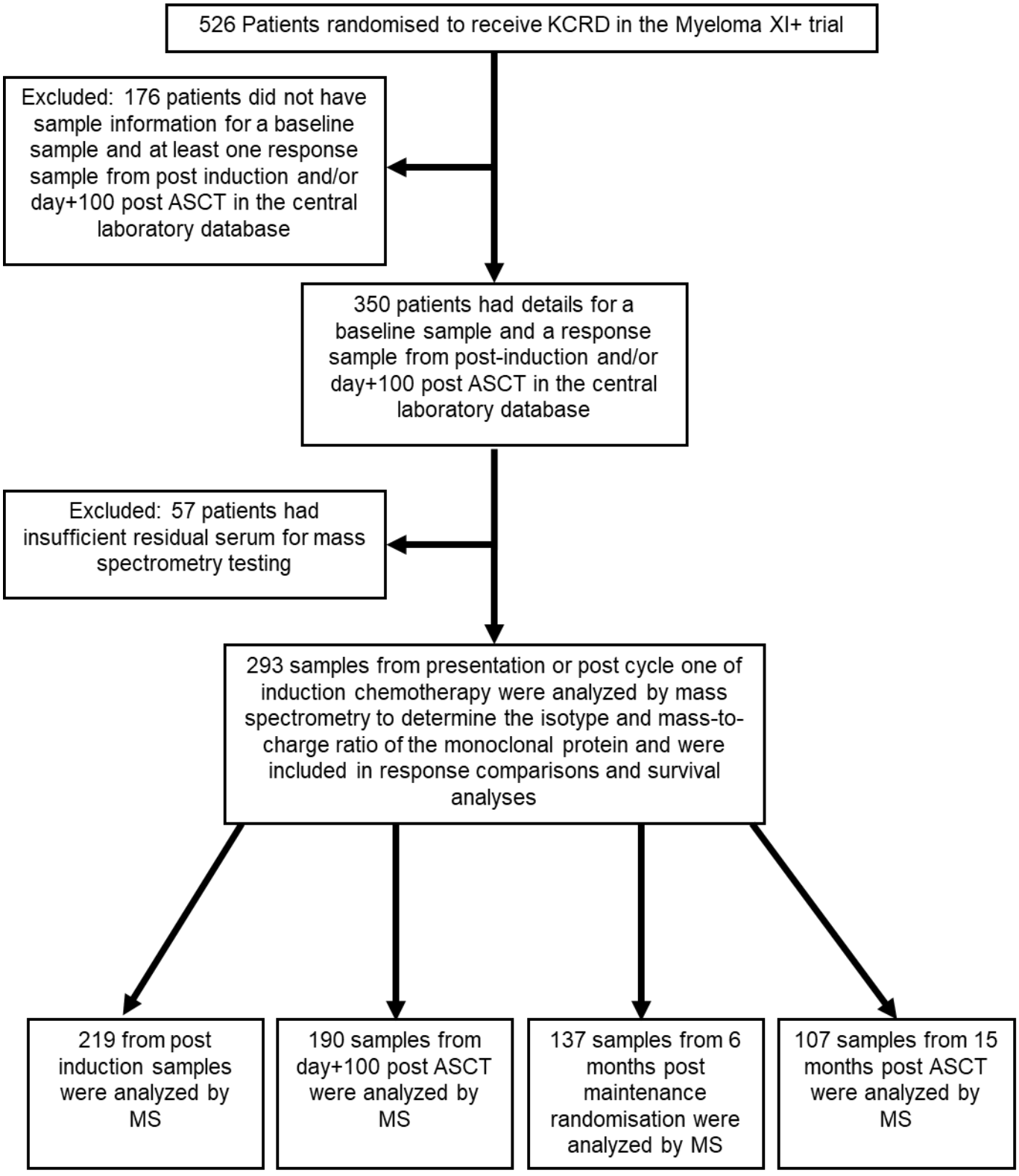


Figure 4. 1 CONSORT Diagram showing the serum samples analysed by MALDI-TOF MS from the Myeloma XI+ trial

The baseline characteristics of the 293 patients included in this study are listed in Table 4.1. 291/293 (99.3%) patients were classified as having secretory disease on the central laboratory analysis, 1/293 (0.3%) was classified as having NSMM and 1/293 (0.3%) had missing central laboratory isotype results. 243/292 (83.2%) patients with available central laboratory results had an intact immunoglobulin monoclonal protein detectable by IFE: 169/243 (69.5%) had an IgG monoclonal protein; 71/243 (29.2%) had an IgA monoclonal protein; and 3/243 (1.2%) had an IgD monoclonal protein. The median follow-up duration for patients included in this study was 43.7 months.

Table 4. 1 Baseline characteristics of the patients included in this study

Patient Characteristic	All Patients (n=293)
Median age, years	60.2
<65 years, n (%)	211 (72.0)
≥65 years, n (%)	82 (28.0)
ISS 1, n (%)	132 (45.1)
ISS 2, n (%)	104 (35.5)
ISS 3, n (%)	51 (17.4)
missing, n (%)	6 (2.0)
Median serum albumin, g/L (range)	40 (18.7-52.6)
Median beta-2-microglobulin, mg/L (range)	3.4 (1.1-30.1)
Median serum creatinine, μmol/L (range)	93 (42-315)
Missing	2 (0.7)
IgG, n (%)	169 (57.7)
IgA, n (%)	71 (42.0)
FLC only, n (%)	48 (16.4)
IgD, n (%)	3 (1.0)
NSMM, n (%)	1 (0.3)
Missing (%)	1 (0.3)
Kappa light chain, n (%)	187 (63.8)
Lambda light chain , n (%)	105 (35.8)
NSMM, n (%)	1 (0.3)
Intact immunoglobulin MP level, g/L (range)	33.2 (0.27-101.7)
iFLC, mg/L (range)	435 (0.37-40347)
Received ASCT	
Yes, n (%)	259 (88.4)
No, n (%)	34 (11.6)
Maintenance Randomisation	
Lenalidomide, n (%)	153 (52.2)
Observation, n (%)	74 (25.3)
Not randomised, n (%)	66 (22.5)

4.3.2 Detection of Monoclonal Protein in Patients with Newly Diagnosed Multiple Myeloma by EXENT and FLC-MS

The isotype of the monoclonal protein identified by IFE and MALDI-TOF MS are shown in Table 4.2. MALDI-TOF MS identified monoclonal protein with the same heavy chain isotype as identified by IFE in 238/240 (99.2%) samples from patients with IgG and IgA monoclonal proteins. Both patients with discrepant heavy chain isotype results had predominantly FLC secreting disease. The first patient had an IgA kappa monoclonal protein detectable by IFE (0.27 g/L IgA kappa monoclonal protein, serum free kappa 3427 mg/L, serum FLC ratio 2347.3, BJP 0.16 g/g creatinine) and had a 2.2 g/L IgG kappa monoclonal protein detectable by MALDI-TOF MS. The second patient had a non-quantifiable IgG kappa monoclonal protein detectable by IFE with high levels of kappa FLC (serum free kappa 1885 mg/L, serum FLC ratio 213.2 and BJP 2.34 g/L) and by MALDI-TOF MS the monoclonal protein identified was kappa light chain only.

6/48 (12.5%) of patients classified as having FLC only myeloma using IFE and serum FLC assessments had an intact immunoglobulin monoclonal protein detectable by MALDI-TOF MS with the same light chain isotype of the monoclonal FLC. In 5/6 (83.3%) patients the monoclonal FLC and intact immunoglobulin monoclonal protein had the same *m/z*, suggesting that these patients may have progressed to multiple myeloma from a low-level intact immunoglobulin MGUS rather than a pure FLC only MGUS.

Table 4. 2 Comparison between the heavy chain and light chain isotypes of the monoclonal protein identified at baseline by IFE versus MALDI-TOF MS

Isotype of the monoclonal protein identified by MALDI-TOF MS	Isotype of the monoclonal protein identified by IFE								
	IgGK	IgGL	IgAK	IgAL	FK	FL	IgDL	NS	Missing
IgGK	117		1		2				1
IgGL		51				2		1	
IgAK			38						
IgAL				31	1	1			
FK	1				25				
FL						17	3*		
IgDL									
NS									
Baseline sample only run against heavy chain beads			1**						

**No IgD beads were available for MALDI-TOF MS testing*

*** the sample was not run against the light chain beads due to limited volume but the same heavy chain isotype for the monoclonal protein was identified by MALDI-TOF MS*

Monoclonal protein with the same light chain isotype as reported by the central laboratory analysis was detectable by the combined panel of QIP-MS/EXENT and FLC-MS in 290/291 (99.3%) patients. In the patient where the results were discrepant, the patient was IFE negative but had an abnormal sFLC ratio with low-level elevation of the serum free kappa levels (serum free kappa 46.64 mg/L, serum free lambda 13.07 mg/L, sFLC ratio 3.57). No BJP, immunophenotyping or immunohistochemistry results were available for comparison. MALDI-TOF MS identified a small 0.025 g/L IgA lambda monoclonal protein in this sample and no monoclonal FLC. The reason for the isotype discrepancies is unclear but the overall rate of discrepant results is similar to those reported in previous MALDI-TOF MS studies of patients with PCD (Mills *et al*., 2016, Puig *et al*., 2022).

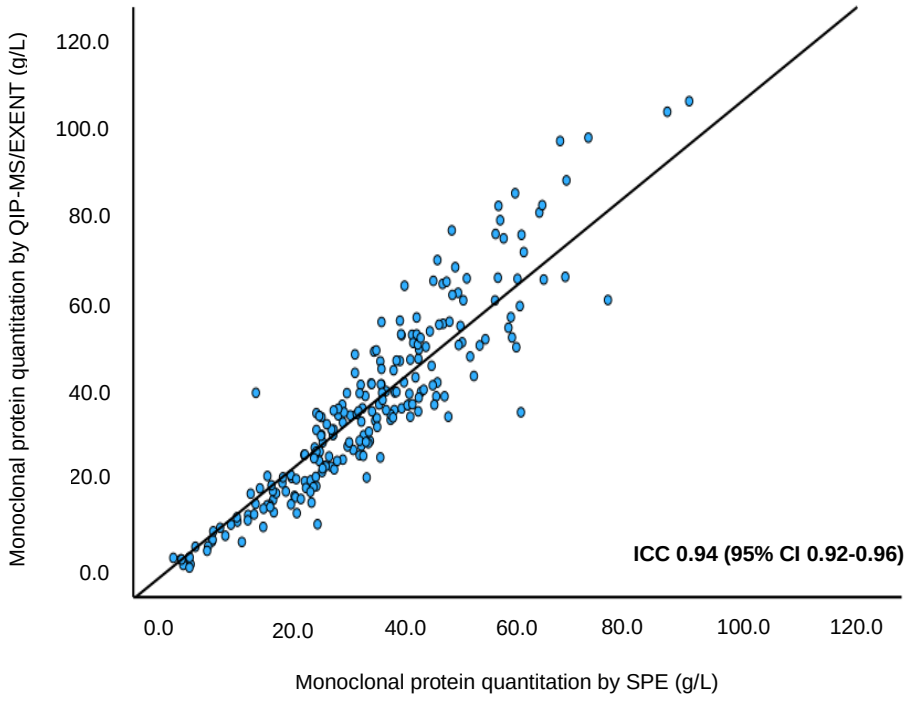


Figure 4. 2 Agreement between monoclonal protein quantitation by SPE and MALDI-TOF MS in patients with newly diagnosed multiple myeloma from the KCRD arm of the Myeloma XI trial assessed by ICC

The monoclonal protein values obtained by QIP-MS/EXENT and SPE from the central laboratory testing performed in the Myeloma XI trial are shown. The agreement between the quantitative results was assessed by ICC and the 95% CI interval is shown.

Overall there was good agreement the monoclonal protein quantitation results obtained by MALDI-TOF MS and SPE (ICC 0.94 (95% CI 0.92-0.96)). However, at very high monoclonal levels there was a tendency for the reported size of the monoclonal protein to be greater by MALDI-TOF MS than SPE. This may be due to overestimation of the total immunoglobulin levels by the immunochemical used to measure the total immunoglobulin levels (Riches *et al*., 1991), which is used to quantitate the monoclonal proteins identified by the QIP-MS-EXENT assay. Underestimation of some of the high-level IgG monoclonal proteins by SPE due to dye saturation (Murray *et al*., 2009) may have also contributed to the slightly greater differences in the size of the larger monoclonal proteins quantitated by MS in comparison to SPE.

4.3.3 Detection and Prognostic Significance of Residual Monoclonal Protein Detectable by MALDI-TOF MS Compared to Achieving IFE Negativity and Bone Marrow-based MRD Assessments at the End of Induction Chemotherapy in Patients Treated with KCRD in the Myeloma XI Trial

219 samples from post induction were available for MALDI-TOF MS testing: 83/219 (37.9%) samples were from patients who still had detectable residual monoclonal protein by SPE and/or IFE; 135/219 (61.6%) patients had no residual monoclonal protein detectable by IFE; and 1/219 (0.5%) had missing central response results.

81/83 (97.6%) patients with residual monoclonal protein detectable by IFE had residual monoclonal protein detectable by MALDI-TOF MS. The details of the two samples in which there was residual monoclonal protein reported on SPE but there was residual monoclonal protein detectable by MALD-TOF MS are listed in Table 4.3.

Both patients were MRD negative by flow cytometry at the end of induction chemotherapy, had normal iFLC levels and serum FLC ratios and remain progression free, suggesting that the residual monoclonal protein reported by SPE may have been false positive results due to oligoclonal bands.

Table 4. 3 Patients with residual monoclonal protein reported in their post-induction sample using SPE/IFE in whom there was no residual monoclonal protein detectable by MS

Monoclonal protein Isotype	Centrally Assessed Response	SPE (g/L)	iFLC (mg/L)	sFLC ratio	MRD	MS Result
GK	VGPR	1.0	11.5	1.39	Negative	Oligoclonal IgA and IgM spectra
GK	VGPR	0.9	5.7	0.87	Negative	Oligoclonal IgM spectrum

There was excellent agreement between the monoclonal protein quantitation results (Figure 4.3A) and monoclonal protein responses obtained by QIP-MS/EXENT and SPE (Figure 4.3B).

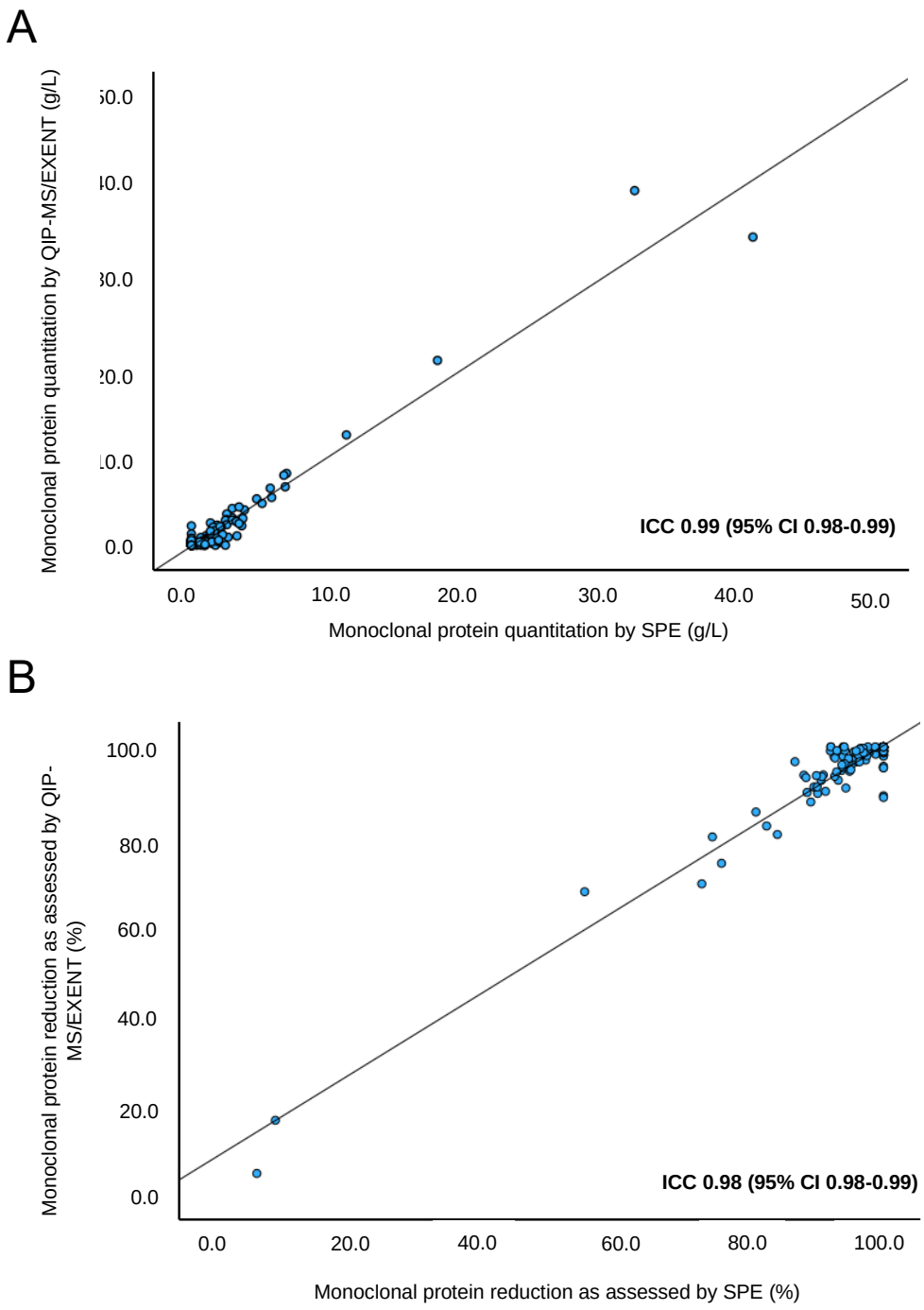


Figure 4. 3 ICC of the monoclonal protein quantitation and response assessments at the end of induction chemotherapy by QIP-MS/EXENT and SPE in patients with intact immunoglobulin secreting multiple myeloma

(A) Shows the ICC of the quantitation of the residual monoclonal protein at the end of induction chemotherapy by SPE and QIP-MS/EXENT. (B) shows the ICC of the monoclonal protein response at the end of induction as assessed by SPE and QIP-MS/EXENT.

The absence of detectable monoclonal protein MALDI-TOF MS at the end of induction chemotherapy was associated with improved PFS (median PFS NR v. 50.0 months, p=0.008; Figure 4.4B). However, the absence of detectable residual monoclonal by IFE at the end of induction was only associated with a non-statistically significant trend towards improved PFS (median PFS NR v. 50.0 months, p=0.075; Figure 4.4A).

102/219 (46.6%) patients had MRD results available from the end of induction chemotherapy; 62/102 (60.8%) were MRD negative by flow cytometry and 40/102 (39.2%) were MRD positive. Patients who were MRD negative at the end of induction had significantly longer PFS than MRD positive patients (median PFS 38.7 months v. NR for MRD positive v negative at the end of induction, p<0.001). There was agreement between bone marrow MRD and IFE and MALDI-TOF MS results in 69/102 (67.6%) and 59/102 (57.8%) of samples respectively (Figure 4.4E). The majority of discrepancies between bone marrow MRD and IFE were due to MRD positivity in the absence of detectable residual disease. In contrast most of the discrepant results between bone marrow MRD and MALDI-TOF MS assessments were due to residual positivity by MALDI-TOF MS in the absence of detectable MRD by flow cytometry.

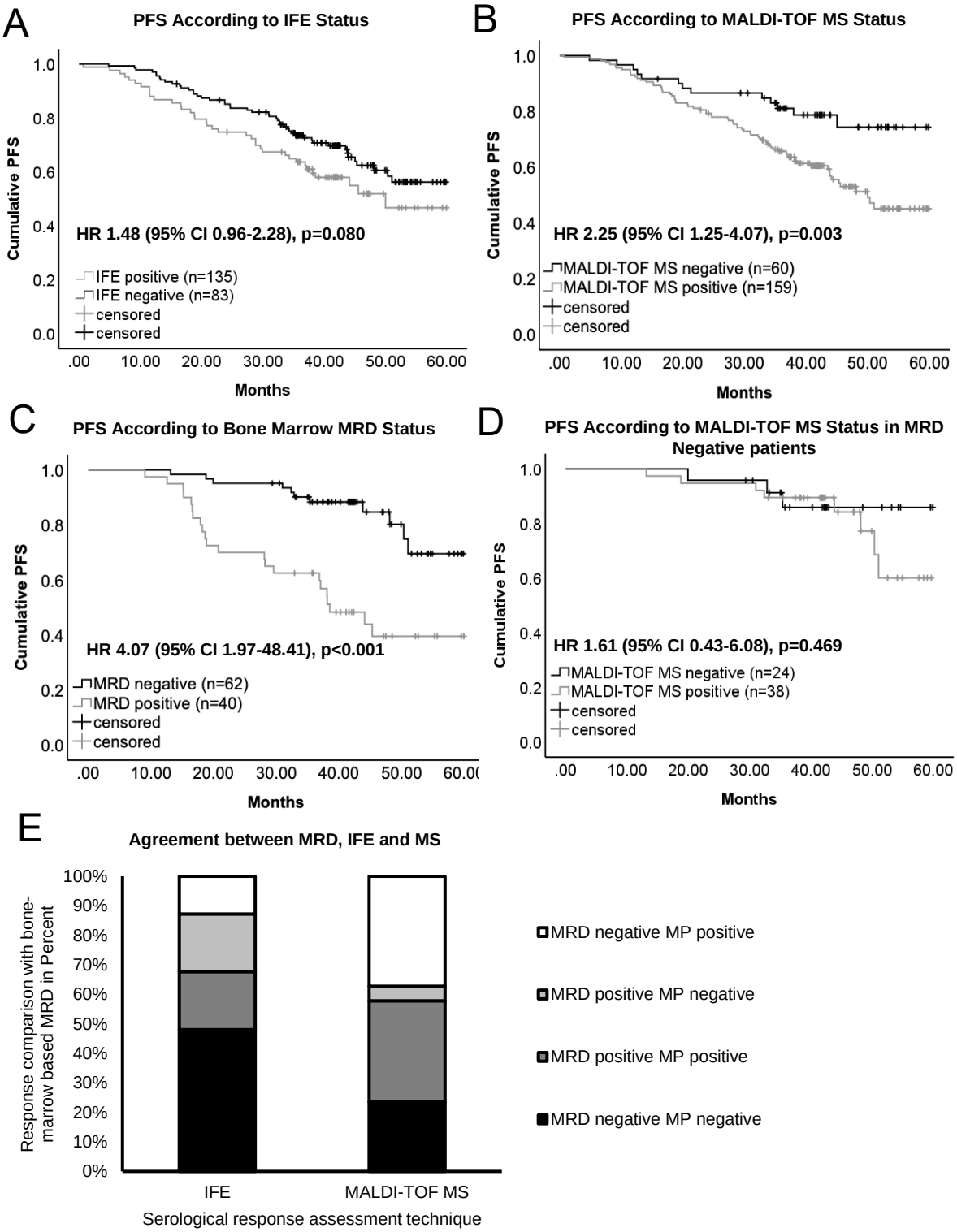


Figure 4. 4 PFS based on depth of response at the end of induction chemotherapy and agreement between MS an bone marrow-based MRD assessments

PFS according to IFE status (A), MALDI-TOF MS-based response assessment (B) and bone marrow MRD assessment (C) at the end of induction chemotherapy. (D) shows PFS according to MALDI-TOF status in patients who were MRD negative at the end of induction. (E) shows the agreement between bone marrow MRD, IFE and MALDI-TOF MS.

Amongst patients who were MRD negative by flow cytometry there was no statistically significant difference in the PFS on the basis of MALDI-TOF MS status at the end of induction (median PFS NR v. NR, p=0.479; Figure 4.4D). On multivariate analysis MRD negativity at the end of induction retained its prognostic significance for PFS (Table 4.4). MALDI-TOF MS only retained it prognostic significance for PFS on multivariate analysis when measures of end of induction response were included in the model.

Table 4. 4 Univariate and multivariate analysis of factors from presentation and the end of induction chemotherapy impacting PFS

Parameter	Univariate for PFS			Multivariate for PFS			Multivariate model 2 for PFS		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Haemoglobin <100g/L	1.72	1.10-2.68	0.018	0.99	0.98-1.01	0.224			
non-IgG monoclonal protein	0.92	0.59-1.42	0.697						
ISS >1	1.09	0.71-1.68	0.68						
Serum creatinine at presentation (µmol/L)	1.01	1.00-1.01	0.024	1.02	1.01-1.03	0.002			
Age	1.04	1.01-1.07	0.009	1.03	0.98-1.08	0.308			
IFE positive at the end of induction	1.49	0.97-2.30	0.069						
MALDI-TOF MS positive at the end of induction	2.25	1.25-4.07	0.007				3.53	1.07-11.71	0.039
MRD positive at the end of induction	4.07	1.97-8.41	<0.001	4.32	1.98-9.43	<0.001	3.50	1.68-7.29	<0.001

4.3.4 Detection and Prognostic Significance of Disease Detectable by MALDI-TOF MS and Bone Marrow-based MRD in Patients with no Detectable Monoclonal Protein by IFE at the End of Induction Chemotherapy in Patients Treated with KCRD in the Myeloma XI Trial

78/135 (57.8%) samples from patients with no residual monoclonal protein detectable by IFE had residual monoclonal protein detectable by MALDI-TOF MS (Figure 4.5A). Example mass spectra from a patient who was IFE negative but had residual monoclonal protein detectable by MALDI-TOF MS are shown in Figure 4.5B). 20/78 (25.6%) samples that were positive by MALDI-TOF MS only had residual monoclonal protein detectable by FLC-MS (Figures 4.5C-E). 9/12 (75.0%) patients with FLC-only secreting disease with residual monoclonal protein detectable by MALDI-TOF MS only had residual monoclonal protein detectable by FLC-MS (Figure 4.5E).

The rate of residual monoclonal protein detection by MALDI-TOF MS was significantly higher in patients with IgG multiple myeloma compared to patients with non-IgG multiple myeloma (77.6% v 44.4%, $p<0.001$). However, there was no difference in the proportion of patients with residual MRD detectable in patients with IgG versus non-IgG multiple myeloma (27.6% v. 28.2%, $p=0.828$). These results suggest that the increased rate of positivity for residual monoclonal protein by MALDI-TOF MS observed in patients with IgG myeloma was related to the longer half-life of IgG compared to IgA and FLC.

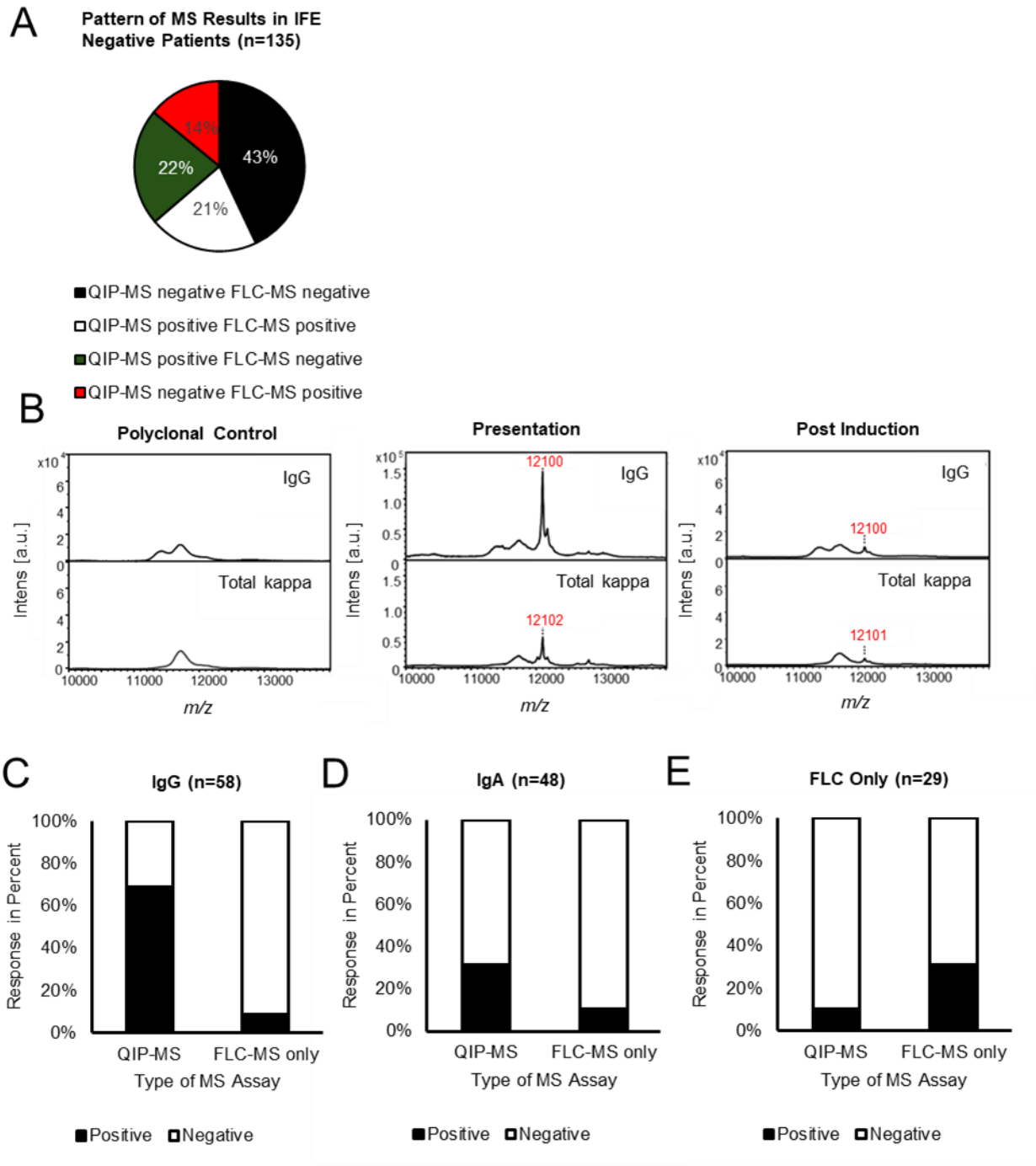


Figure 4. 5 Monoclonal protein detection rates by MS in patients negative for residual monoclonal protein by IFE post induction.

(A) shows the monoclonal protein detection rate by MS in all IFE negative patients. (B) shows example mass spectra from a patient with IgG kappa myeloma who had no residual monoclonal protein detectable by SPE or IFE at the end of induction chemotherapy but had a 297 mg/L residual IgG kappa monoclonal protein detectable by QIP-MS. The monoclonal protein peaks are highlighted by the red mass labels. (C-E) show the proportion of patients with residual monoclonal protein detectable by QIP-MS/EXENT and patients who only had residual monoclonal protein detectable with addition of FLC-MS in patients with IgG (n=58), IgA (n=48) and FLC (n=29) myeloma respectively.

When the analysis was restricted only to patients in whom there was no residual monoclonal protein detectable by IFE, MRD negativity and MALDI-TOF MS negativity were both still associated with improved PFS (Figure 4.6).

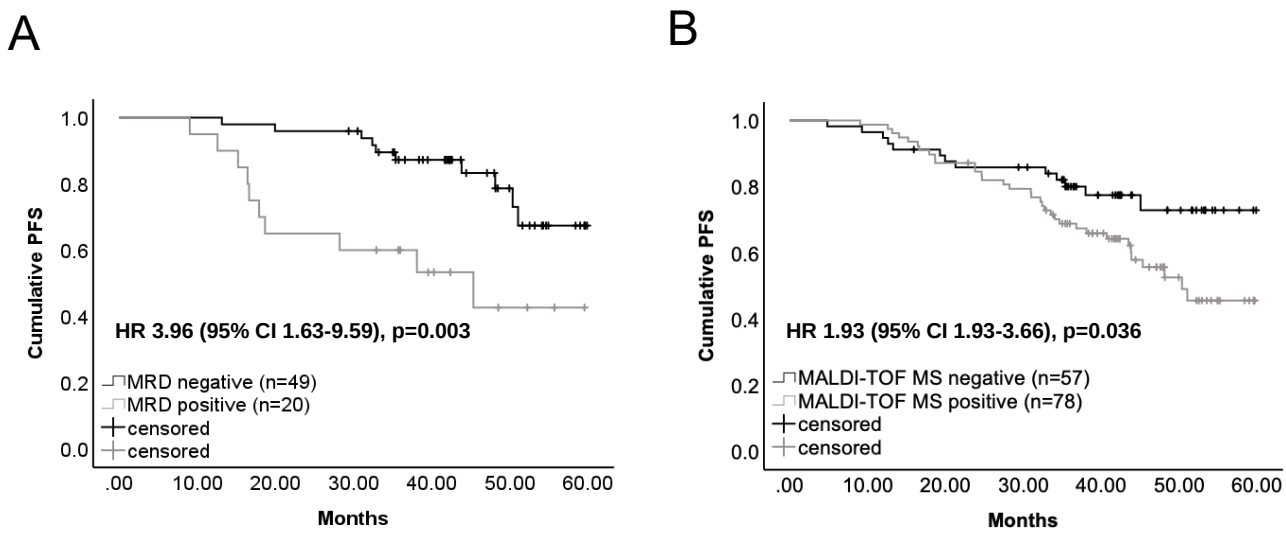


Figure 4. 6 PFS according to bone marrow MRD and MALDI-TOF MS-based response assessments in patients treated with KCRD in the Myeloma XI trial who had no residual monoclonal protein detectable by IFE at the end of induction

The PFS according to bone marrow flow cytometry MRD assessment is shown in (A) and the PFS according to combined MALDI-TOF MS response using QIP-MS and FLC-MS is shown in (B).

4.3.5 Sensitivity and Prognostic Implications of FLC-MS for the Detection of Residual Monoclonal FLC Compared to FLC Assessments in Patients treated with KCRD in the Myeloma XI Trial with No Residual Monoclonal Protein detectable by IFE at the End of Induction Chemotherapy

32/135 (23.7%) patients with no residual monoclonal protein detectable by IFE had an abnormal sFLC ratio and 47/135 (34.8%) had residual monoclonal FLC detectable by FLC-MS. Overall, 53/135 (39.3%) patients had discrepant results; 34/53 (64.2%) had residual monoclonal FLC detectable by FLC-MS but had a normal FLC ratio and 19/53 (35.8%) had no detectable residual monoclonal FLC by FLC-MS but had an abnormal serum FLC ratio (Figure 4.7). Concordance between FLC-MS and Freelite®

assessments was significantly higher for patients with an abnormal FLC ratio with an increased iFLC compared to patients with an abnormal FLC ratio with supressed uFLC levels (70.0% v. 25.0%, p=0.023).

FLC-MS positivity in patients with no residual monoclonal detectable by IFE at the end of induction was associated with shorter PFS (median PFS 43.50 months v. NR, p<0.001; Figure 4.7C). The prognostic significance of FLC-MS status the end of induction was retained on multivariate analysis (Table 4.5). However, there was no significant difference between the PFS of patients with and without an abnormal serum FLC ratio (median PFS 43.86 months v. NR, p=0.17; Figure 4.7D). Even when patients with abnormal FLC ratio due to suppression of the uFLC were removed from the abnormal FLC ratio cohort (Figure 4.7F) and when only patients with an abnormal FLC ratio due to a persistently elevated iFLC level were categorised as having an abnormal serum FLC ratio, Freelite® still did not differentiate two groups with statistically different PFS (Figure 4.7E).

The lack of prognostic significance even after removal of patients with abnormal FLC ratios due to suppression of the uFLC suggests that additional factors other than treatment-related immune suppression, such as limited sensitivity, may also be contributory factors to the lack of prognostic significance of FLC ratio.

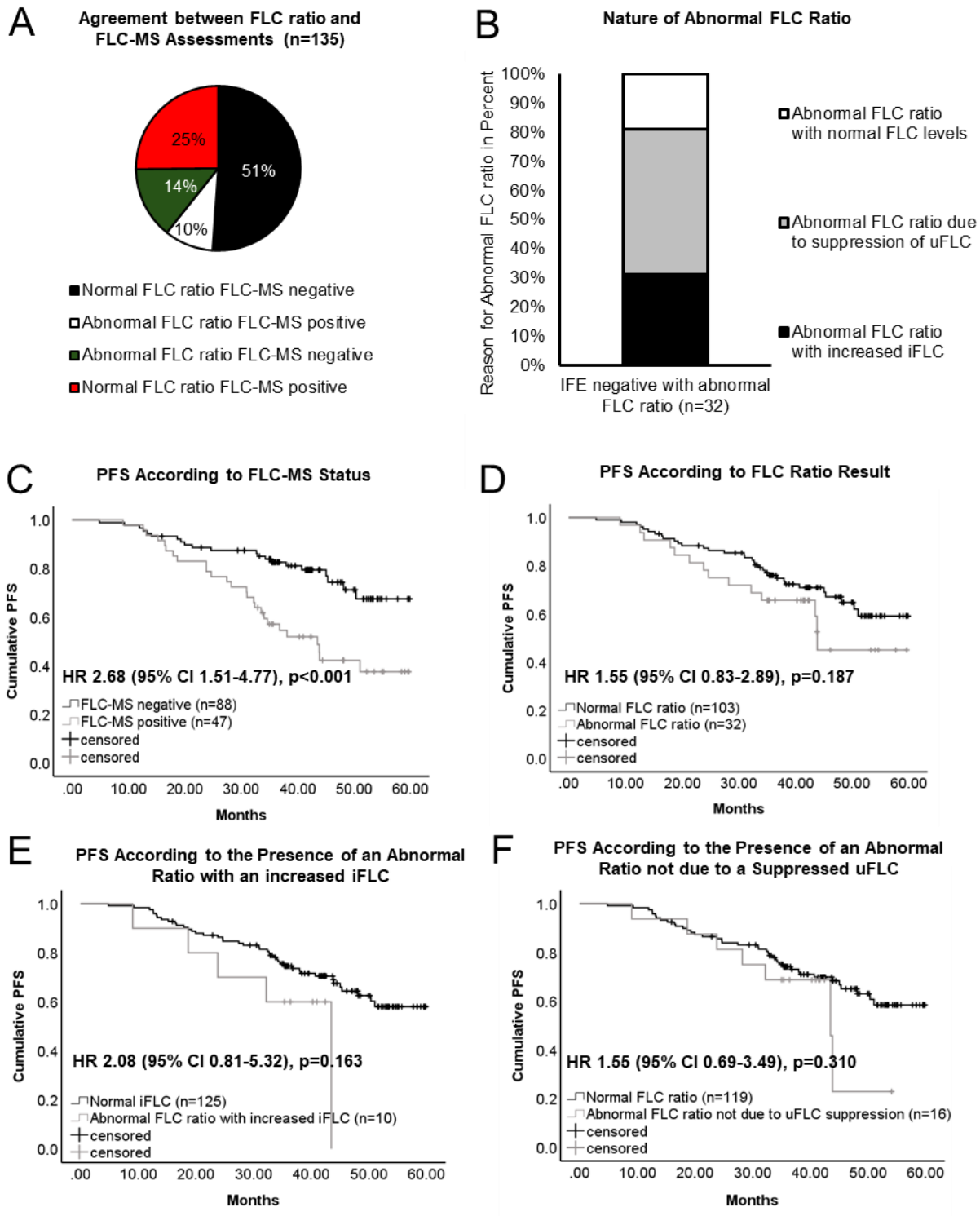


Figure 4. 7 Agreement between FLC ratio and FLC-MS assessments at the end of KCRD induction chemotherapy in patients treated in the Myeloma XI trial and their prognostic significance

(A) Shows the agreement between FLC-MS and Freelite® using the normal range for FLC ratio of 0.26-1.65 for patients with eGFR ≥30ml/minute and 0.37-3.1 for patients with eGFR <30 ml/minute and (B) shows the cause of the abnormal FLC ratios. (C) shows the PFS for patients according to FLC-MS status at the end of induction and (D-F) show the PFS according to FLC ratio.

Table 4. 5 Univariate and multivariate analysis of factors affecting PFS in patients who were IFE negative at the end of induction

	n/N	HR	95% CI	*p* value
Univariate analysis				
FLC-MS positive	47/135	2.68	1.51-4.77	<0.001
MRD positive	19/68	3.96	1.63-9.59	0.002
Age	135/135	1.03	1.00-1.07	0.068
ISS >1	66/133	0.90	0.51-1.60	0.72
Non-IgG	77/135	1.29	0.72-2.30	0.40
abnormal serum FLC ratio	32/135	1.55	0.83-2.89	0.17
Multivariate analysis 1				
FLC-MS positive	47/135	4.45	1.64-12.02	0.003
MRD positive	19/68	2.65	1.05-6.68	0.039
Age	135/135			
ISS >1	66/133			
Non-IgG	77/135			
abnormal serum FLC ratio	32/135			
Multivariate analysis 2				
FLC-MS positive	47/135	4.48	1.65-12.18	0.003
MRD positive	19/68	3.24	1.19-9.93	0.022
Age	135/135	1.04	0.97-1.11	0.28
ISS >1	66/133			
Non-IgG	77/135			
abnormal serum FLC ratio	32/135			

4.3.6 Prognostic Significance of Residual Monoclonal Protein Detectable by MALDI-TOF MS Compared to Achieving IFE Negativity and Bone Marrow-based MRD Assessments at Day+100 Post ASCT in patients treated in the KCRD Arm of the Myeloma XI Trial

190 patients had sufficient residual serum from day+100 post ASCT for QIP-MS/EXENT testing and 189/190 (99.5%) also had sufficient residual volume for FLC-MS testing. Residual monoclonal protein was detectable by MALDI-TOF MS in 93/190 (48.9%) patients overall. In 75/190 (39.5%) patients the residual monoclonal protein

was detectable by QIP-MS/EXENT but in 18/189 (9.5%) residual monoclonal protein was only detectable by FLC-MS.

Monoclonal protein was detectable by MALDI-TOF MS in 35/38 (92.1%) patients with residual detectable using standard electrophoretic techniques. Similar to the discrepant results observed at the end of induction chemotherapy, the monoclonal proteins detected by SPE in patients who had no residual monoclonal protein detectable by MALDI-TOF MS were all relatively small (0.99-2.7 g/L). MRD results were only available 1/3 (33.3%) patients of these patients and they were MRD negative.

There was agreement between response assessments by MALDI-TOF MS and bone marrow MRD by flow cytometry in 94/140 (67.1%) of patients. The majority of discrepant results were due to MS positivity and bone marrow negativity (Figure 4.8D). Residual monoclonal FLC were detectable by MS in 29/41(70.7%) samples that were MS positive but MRD negative by flow cytometry, suggesting that the discrepant results at this time point were not all due to the long half-life of intact immunoglobulin monoclonal proteins in many of these patients.

At day+100 post ASCT IFE positivity, MALDI-TOF MS positivity and bone MRD positivity were all associated with reduced PFS (median PFS 38.6 months v.NR, $p<0.01$; median PFS 45.6 months versus NR, $p<0.001$; and median PFS 40.7 months versus NR, $p<0.001$ for positivity for residual disease by IFE, MALDI-TOF MS and bone marrow MRD respectively, Figure 4.8A-C). On multivariate analysis positivity

by MRD and MALDI-TOF MS retained their prognostic significance for PFS but IFE positivity did not (Table 4.6).

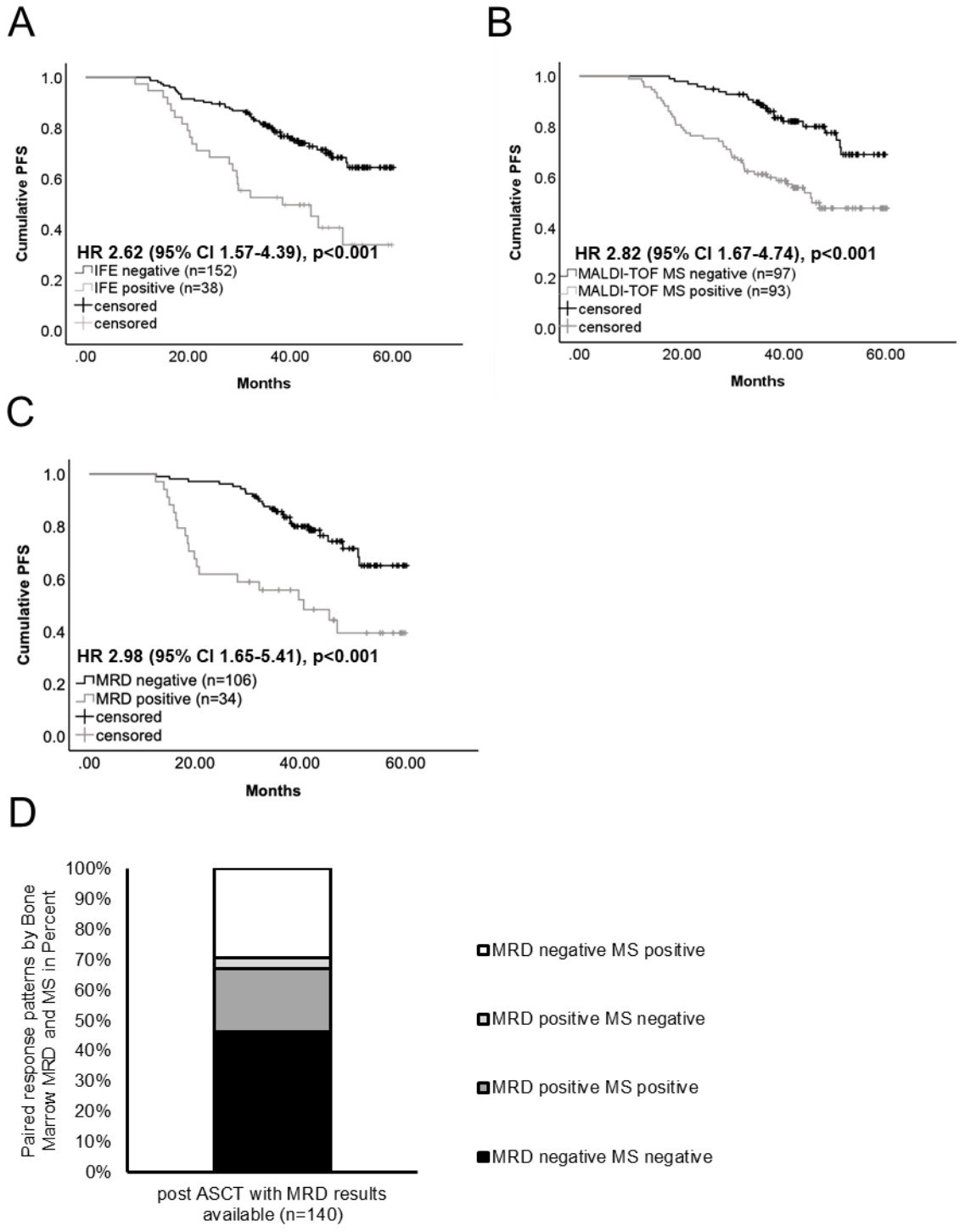


Figure 4. 8 Prognostic significance of IFE, MALDI-TOF MS and bone marrow MRD assessment at day +100 post ASCT.

The PFS according to IFE, MALDI-TOF MS and bone marrow MRD status are shown in (A-C). (D) shows the agreement between bone marrow MRD and MALDI-TOF MS response assessments at day+100 post ASCT in patients with paired results.

Table 4. 6 Univariate and multivariate analysis of variables impacting PFS at day+100 post ASCT

	Univariate for PFS			Multivariate for PFS		
Parameter	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Haemoglobin <100g/L	1.87	1.13-3.09	0.015	1.88	1.01-3.50	0.048
non-IgG monoclonal protein	0.63	0.37-1.06	0.080			
ISS >1	1.28	0.78-2.10	0.333			
Serum creatinine at presentation (µmol/L)	1.01	1.00-1.01	0.069			
Age	1.02	0.99-1.05	0.117			
Immunofixation positive at day+100 post ASCT	2.62	1.57-4.39	<0.001	1.00	0.47-2.14	
MALDI-TOF MS positive at day+100 post ASCT	2.82	1.67-4.74	<0.001	2.23	1.09-4.56	0.027
MRD positive at day+100 post ASCT	2.98	1.65-5.41	<0.001	2.33	1.17-4.62	0.016

Good agreement was observed between monoclonal protein quantitation and monoclonal percentage reduction assessments at day+100 post ASCT was observed between SPE and MALDI-TOF MS (Figure 4.9).

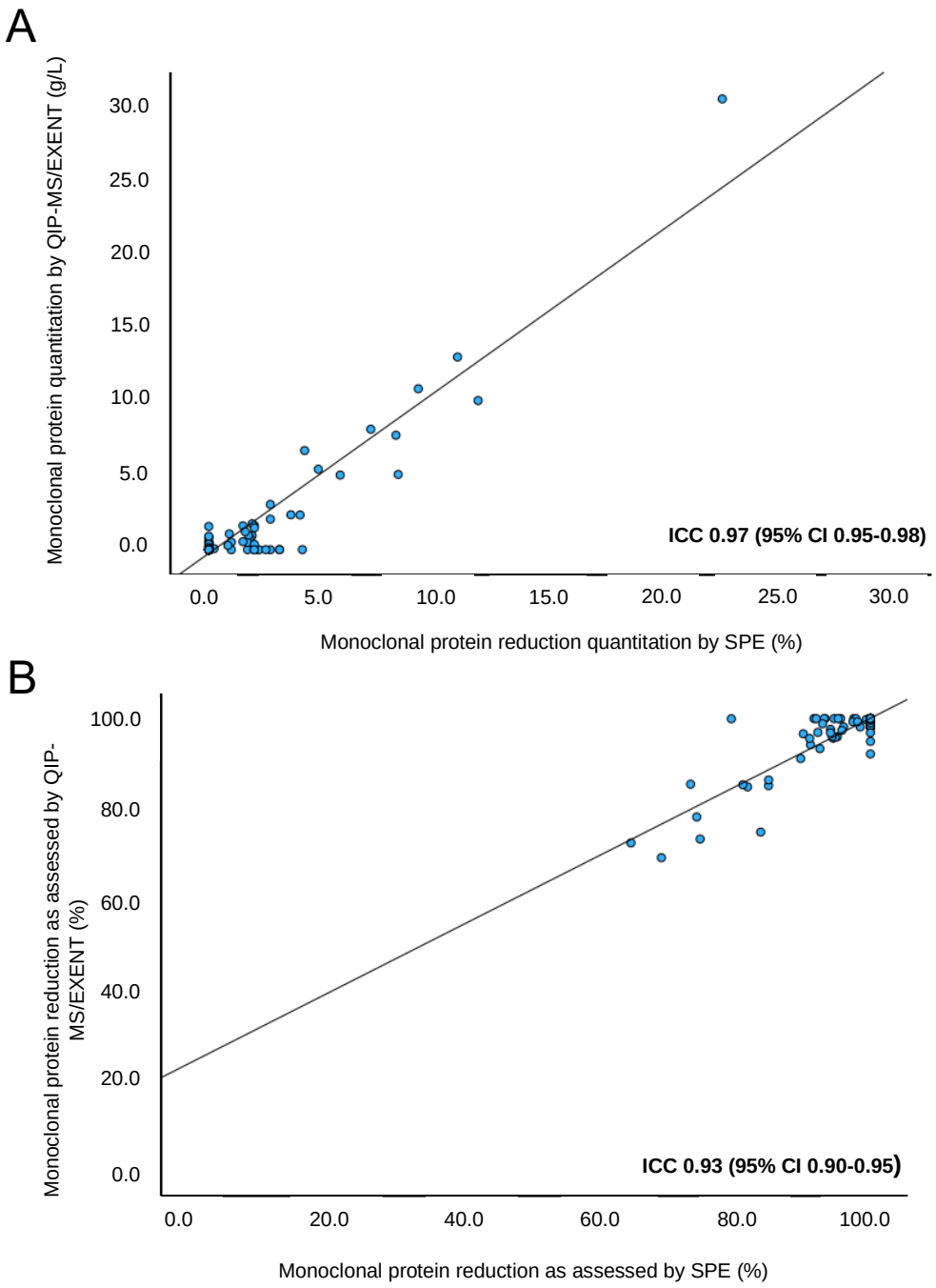


Figure 4. 9 Linear regression plots and ICC of the monoclonal protein quantitation and response assessments at day+100post ASCT by QIP-MS/EXENT and SPE

(A) Shows the agreement between the quantitation of the residual monoclonal protein at the end of induction chemotherapy by SPE and MALDI-TOF MS and (B) shows the agreement between the monoclonal protein response at day+100 post ASCT as assessed by SPE and MALDI-TOF MS.

4.3.7 Detection and Prognostic Significance of Disease Detectable by MALDI-TOF MS and Bone Marrow MRD in Patients in Patients with no Detectable Monoclonal Protein by IFE at Day+100 Post ASCT

Residual monoclonal protein was detectable by MALDI-TOF MS in 58/152 (38.2%) patients who had no residual monoclonal protein detectable by IFE at day +100 post ASCT. 1/152 (0.7%) with no detectable monoclonal protein by IFE, had residual monoclonal protein detectable by EXENT/QIP-MS but there was insufficient residual serum for FLC-MS analysis. In 40/58 (69.0%) samples positive by MALDI-TOF MS, the residual monoclonal was detectable using EXENT/QIP-MS. In the remaining 18 samples residual monoclonal protein was only detectable by FLC-MS (Figure 4.10).

6/14 (42.9%) of the patients with intact immunoglobulin monoclonal protein secreting disease at presentation who only had residual monoclonal protein detectable by FLC-MS have progressed. Serological results from after re-emergence of detectable monoclonal protein using standard techniques were available for 4/6 (66.7%) of these patients and 2/4 (50.0%) relapsed with a light chain escape pattern with increasing iFLC levels but no detectable intact immunoglobulin monoclonal protein. The FLC ratio in the samples from day+100 post ASCT was normal in both patients but 1/2 (50.0%) had detectable MRD in the bone marrow by flow cytometry.

Residual monoclonal protein was detectable by MALDI-TOF MS in 58/152 (38.2%) patients who had no residual monoclonal protein detectable by IFE at day +100 post ASCT. 1/152 (0.7%) with no detectable monoclonal protein by IFE, had residual monoclonal protein detectable by EXENT/QIP-MS but there was insufficient residual serum for FLC-MS analysis. In 40/58 (69.0%) samples positive by MALDI-TOF MS,

the residual monoclonal was detectable using EXENT/QIP-MS. In the remaining 18 samples residual monoclonal protein was only detectable by FLC-MS (Figure 4.10).

6/14 (42.9%) of the patients with intact immunoglobulin monoclonal protein secreting disease at presentation who only had residual monoclonal protein detectable by FLC-MS have progressed. Serological results from after re-emergence of detectable monoclonal protein using standard techniques were available for 4/6 of these patients and 2/4 (50.0%) relapsed with a light chain escape pattern with increasing iFLC levels but no detectable intact immunoglobulin monoclonal protein. The FLC ratio in the samples from day+100 post ASCT was normal in both patients but 1/2 (50.0%) had detectable MRD in the bone marrow by flow cytometry.

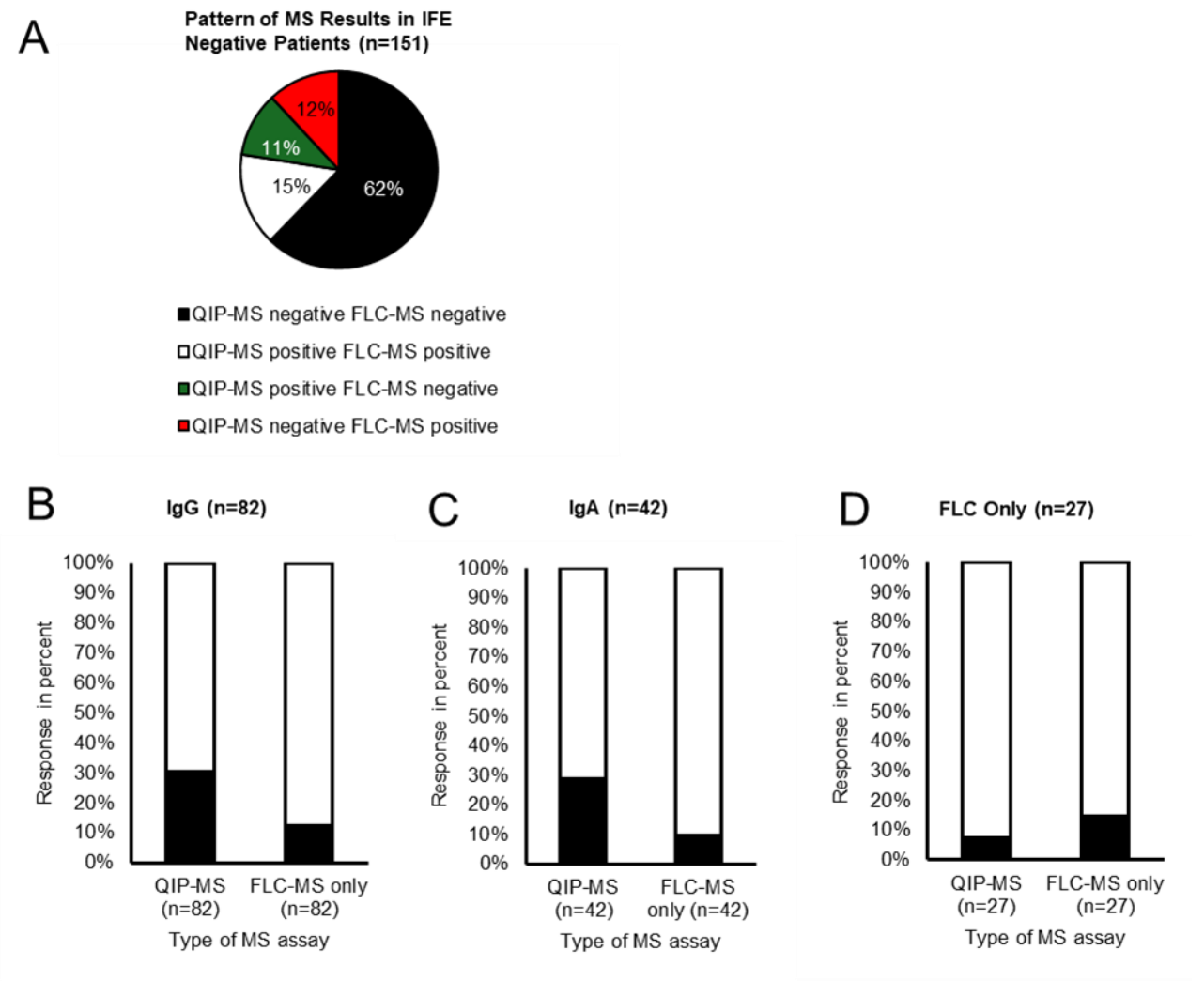


Figure 4. 10 Monoclonal protein detection rates by MALDI-TOF MS in patients with no residual monoclonal detectable by IFE at day+100 post ASCT.
(A) in all IFE negative patients, (B) in IFE negative patients with IgG myeloma, (C) in IFE negative patients with IgA myeloma in and (D) in IFE negative patients with FLC-only myeloma.

When only patients with no residual monoclonal protein by IFE were considered, the presence of residual disease detectable by flow cytometry in the bone marrow and

MALDI-TOF MS in the serum of patients both retained prognostic significance and were associated with reduced PFS (Figure 4.11).

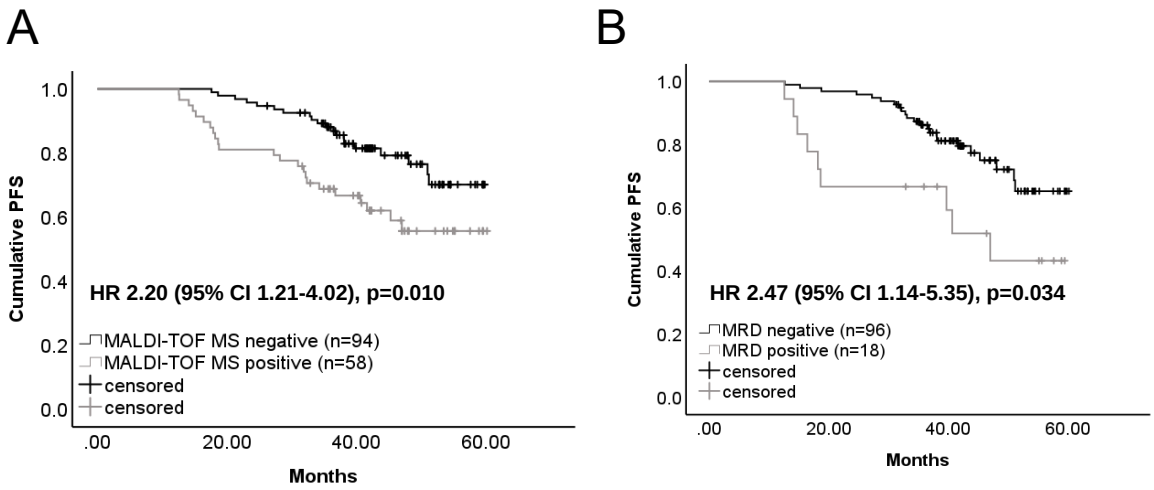


Figure 4. 11 Prognostic significance of residual disease detectable by MALDI-TOF MS and bone marrow MRD at day+100 post ASCT
(A) Shows the PFS according to MALDI-TOF MS status in patients who were IFE negative at day+100 post ASCT. (B) shows the PFS according to bone marrow MRD status at day+100 post ASCT in patients with no detectable residual monoclonal protein by IFE.

**the sample that was only tested by QIP-MS/EXENT has been included in (A)*

4.3.8 Sensitivity and Prognostic Implications of FLC-MS for the Detection of Residual Monoclonal FLC Compared to FLC Assessments at Day+100 Post ASCT

Freelite® results were available for 151/152 (99.3%) patients in whom there was no residual monoclonal protein detectable by IFE at day+100 post ASCT and 150/152 (98.7%) had paired Freelite® and FLC-MS results. 36/150 (24.0%) patients had an abnormal FLC ratio and 41/150 (27.3%) had residual monoclonal FLC detectable by FLC-MS.

Overall, there was agreement between Freelite® and FLC-MS results on 91/150 (60.7%) of patients (Figure 4.12). However, only 9/36 (25.0%) patients with an

abnormal serum FLC ratio had residual monoclonal FLC detectable by FLC-MS. In 12/36 (33.3%) patients the abnormal FLC ratio was due to suppression of the uFLC and only 10/36 (27.8%) patients had an abnormal serum FLC ratio with an increased iFLC level. In patients with an abnormal FLC ratio, patients with kappa iFLC were over-represented; 34/36 (94.4%) patients with an abnormal FLC had kappa light chain secreting disease. This trend was not replicated in the FLC-MS results where both light chain isotypes were much more evenly represented (21/41 (51.2%) and 20/41 (48.8%) had kappa and lambda as the involved light chain at presentation).

Bone marrow MRD results were available for 23/27(85.2%) patients with an abnormal FLC ratio who had no detectable residual monoclonal FLC by MALDI-TOF MS and only 3/23 (13.0%) had MRD detectable by flow cytometry. In 2/3 (66.7%) of these patients the abnormal FLC ratio was due to suppression of the uFLC in the context of globally suppressed polyclonal total immunoglobulins levels.

Residual positivity by FLC-MS at day+100 post ASCT in patients with no detectable residual monoclonal protein by IFE was associated with shorter PFS (Figure 4.12C) and this prognostic significance was retained on multivariate analysis (Table 4.7). However, there was no significant difference in PFS according to FLC ratio status irrespective of whether all patients with an abnormal FLC ratio were considered (Figure 4.12D) or only patients with an abnormal ratio not due to suppression of the uFLC or patients with an abnormal FLC ratio with an increased iFLC level were classified as having an abnormal FLC ratio (Figure 4.12E and F).

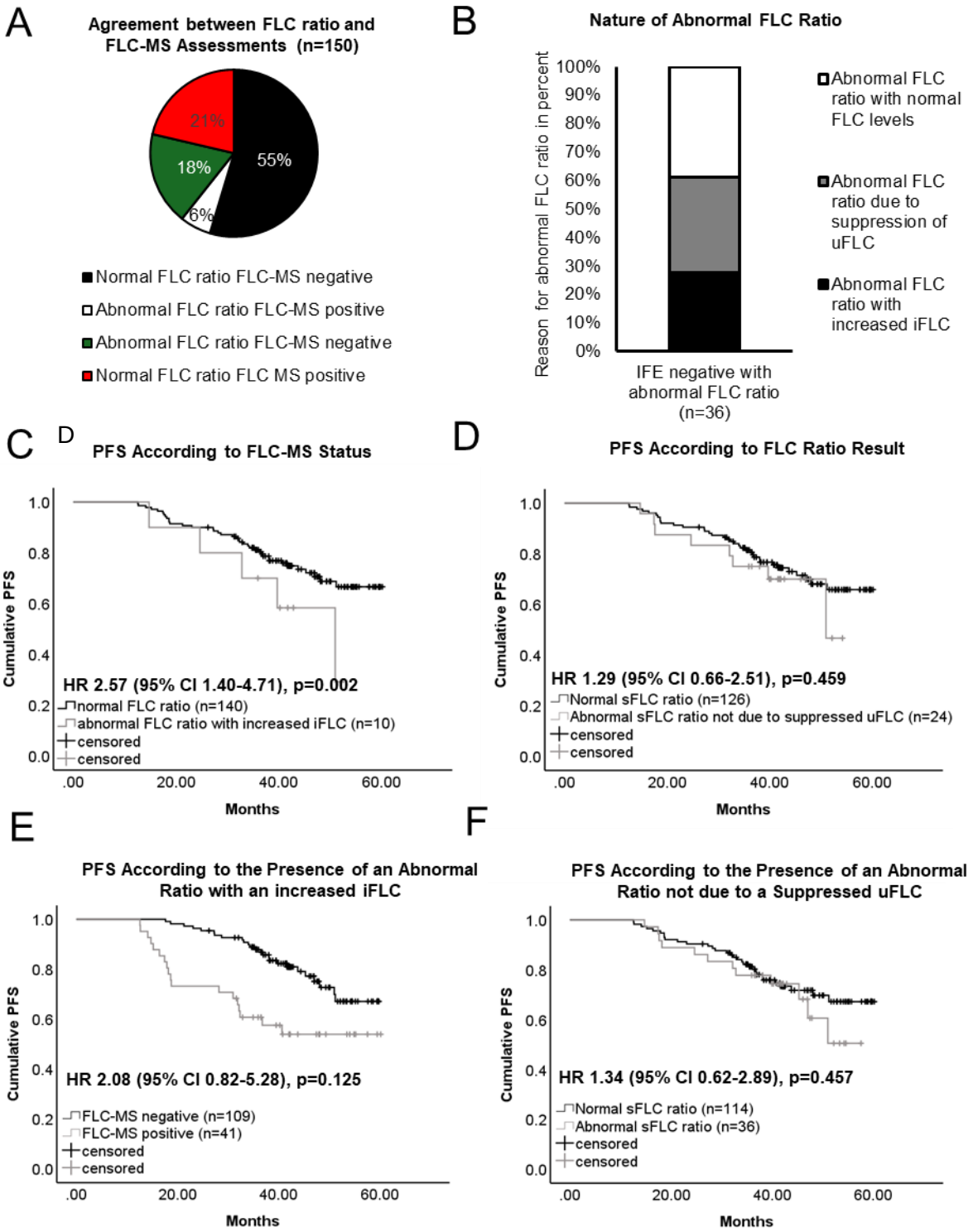


Figure 4.12 Agreement between Freelite® and FLC-MS assessments and their prognostic significance in patients treated with KCRD in the Myeloma XI trial who had no detectable monoclonal protein by IFE at day+100 post ASCT

(A) Shows the agreement between FLC-MS and Freelite® considering the FLC ratio normal if is between 0.26-1.65 for patients with eGFR ≥30ml/minute and 0.37-3.1 for patients with eGFR <30 ml/minute. (B) shows the nature of the abnormal serum FLC ratio results. (C) shows the PFS for patients according to FLC-MS status at day+100 post ASCT and (D-F) show the PFS according to serum FLC ratio results.

Table 4. 7 Univariate and multivariate analysis of factors from presentation and day+100 post ASCT impacting PFS

	n/N	Hazard ratio	95% CI	*p* value
Univariate analysis				
FLC-MS positive	41/150	2.59	1.41-4.75	0.002
MRD positive	17/112	2.74	1.27-5.93	0.01
Age	150/150	1.03	0.99-1.07	0.08
ISS 2/3	80/150	1.31	0.71-2.40	0.38
Non-IgG	69/150	1.04	0.57-1.90	0.91
abnormal sFLC ratio	36/150	1.254	0.64-2.44	0.51
Multivariate analysis 1				
FLC-MS positive	41/150	2.64	1.30-5.38	0.008
MRD positive	17/112	2.33	1.07-5.11	0.034
Age	150/150	NI		
ISS 2/3	80/150			
Non-IgG	69/150			
abnormal sFLC ratio	36/150			
Multivariate analysis 2				
FLC-MS positive	41/150	2.44	1.18-5.02	0.016
MRD positive	17/112	2.77	1.23-6.24	0.014
Age	150/150	1.05	1.00-1.10	0.049
ISS 2/3	80/150			
Non-IgG	69/150			
abnormal sFLC ratio	36/150			

NI: not included

4.3.9 Detection and Prognostic Significance of Residual Monoclonal Protein Detectable by MALDI-TOF MS Compared to Achieving IFE Negativity and BM MRD Assessments at Six Months Post Maintenance Randomisation in Patients treated in the KCRD Arm of the Myeloma XI Trial

136 patients had residual serum from six months post maintenance randomisation. Central laboratory results were available for 118/136 (86.8%) patients. Agreement between IFE and MALDI-TOF MS assessments was observed in 82/118 (69.5%) patients (Figure 4.13A). Residual monoclonal protein was detectable by MALDI-TOF MS in 23/86 (26.7%) patients with no detectable monoclonal protein by IFE (Figure 4.13B).

Bone marrow MRD results were available for 44/136 (32.3%) patients and there was agreement between bone MRD and MALDI-TOF MS assessments in 33/44 (75.0%) patients (Figure 4.13C). 8/11 (72.7%) of the discrepant results were due to MALDI-TOF MS positivity but bone marrow MRD negativity. 7/8 (87.5%) of the patients with discrepant results had intact immunoglobulin monoclonal protein secreting disease and of the seven patients with intact immunoglobulin monoclonal protein disease who were MS positive but bone marrow MRD negative, 5/7 (71.4%) had residual monoclonal FLC and intact immunoglobulin monoclonal protein detectable. The persistence of detectable monoclonal FLC, which only have a half-life of a few hours, suggests that the residual MS positivity was not purely due to the long half-life of intact immunoglobulin monoclonal proteins.

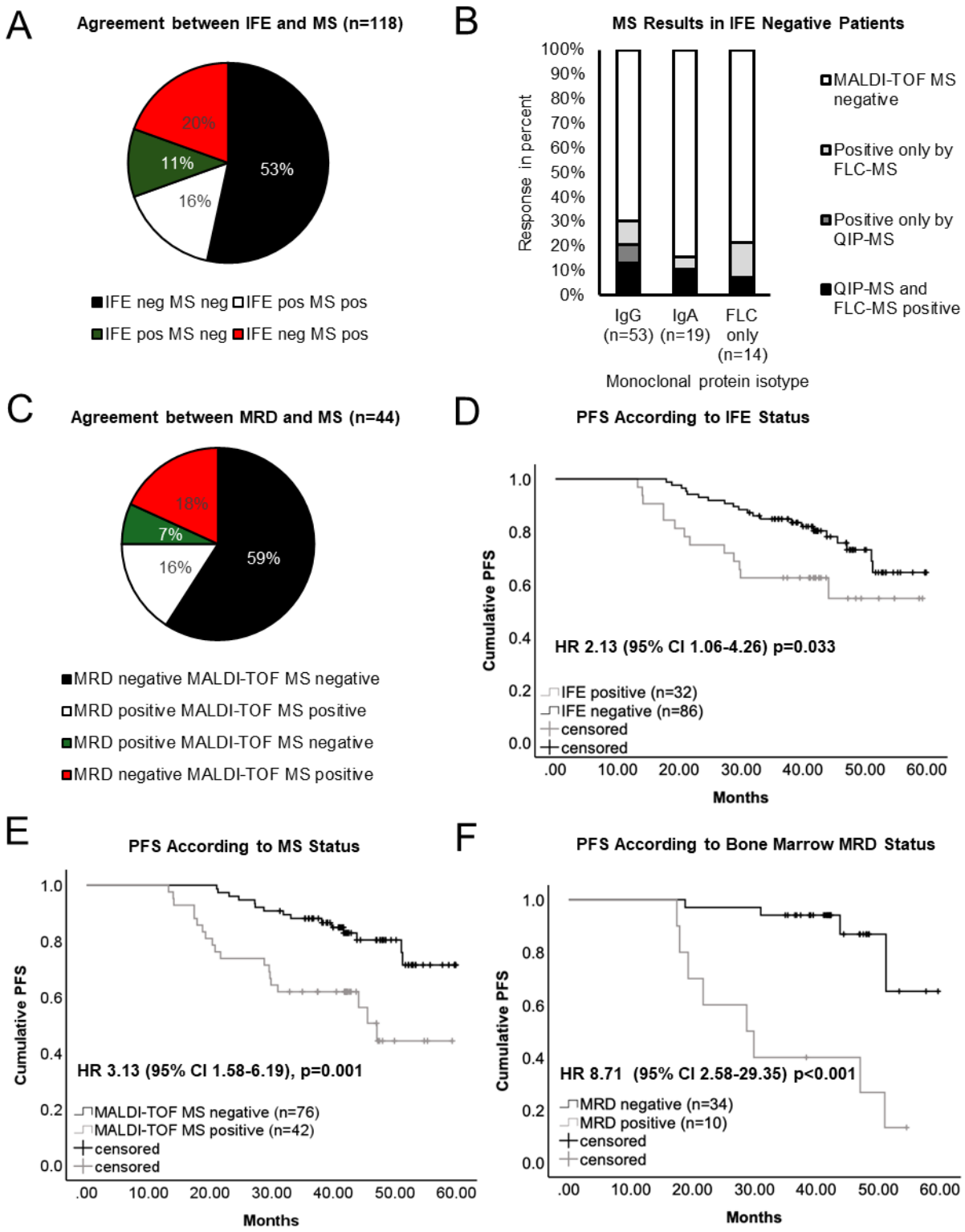


Figure 4. 13 Detection rates of residual disease by MALDI-TOF MS compared to IFE and bone marrow MRD at six months post maintenance randomisation in patients treated in the KCRD arm of the Myeloma XI trial and their prognostic significance

The detection rate of residual disease by MS versus IFE is shown in (A) and compared to MRD in patients with paired results is shown in (C). (B) shows the monoclonal protein detection rate for patients who were IFE negative according to monoclonal protein isotype. (D-F) shows the PFS according to IFE, MS and MRD status at six months post maintenance randomisation.

Positivity for residual disease by IFE, MALDI-TOF MS and bone marrow MRD at six months post maintenance randomisation were all associated with reduced PFS (Figure 4.13D-F).

Patients with no detectable residual monoclonal protein by IFE who had detectable MRD in the bone marrow by flow cytometry had reduced PFS compared to MRD negative patients (median PFS 47.1 months v. NR, p=0.03; Figure 4.14A). However, MALDI-TOF MS positivity in IFE negative patients was not associated with a statistically significant difference in PFS (median PFS 51.1 months v. NR, p=0.08; Figure 4.14B). Longer follow-up is required to determine whether the trend towards shorter PFS in MALDI-TOF MS would become statistically significant as progression events have only occurred in 21/86 (24.4%) patients so far.

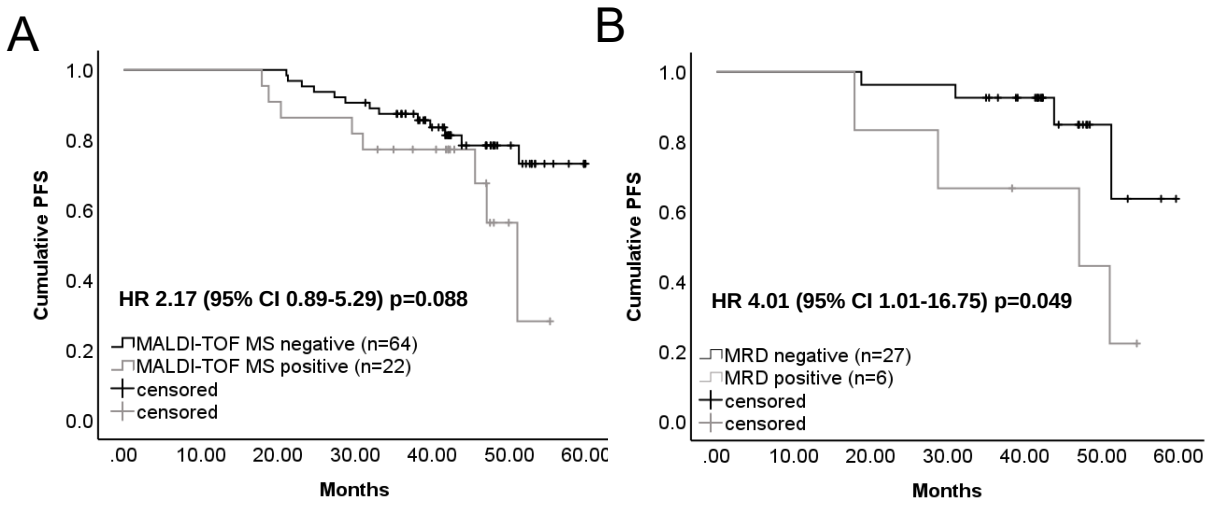


Figure 4. 14 PFS according to residual disease status as assessed by MALDI-TOF MS and flow cytometry in patients with no detectable residual monoclonal protein by IFE at six months post maintenance randomisation

The PFS according to MALDI-TOF MS status in shown in (A) and the PFS according to bone marrow MRD status is shown in (B).

4.3.10 Sensitivity and Prognostic Implications of FLC-MS for the Detection of Residual Monoclonal FLC Compared to serum FLC Assessments at Six Months Post Maintenance Randomisation in Patients treated in the KCRD Arm of the Myeloma XI Trial

Residual monoclonal FLC was detectable by MALDI-TOF MS in 18/86 (20.9%) samples from patients in whom there was no detectable monoclonal protein by IFE at six months post maintenance randomisation. Complete serum FLC ratio and renal function results were available for 58/86 (67.4%) of these patients; 14/58 (24.1%) patients had an abnormal serum FLC ratio and 12/58 (20.7%) patients had residual monoclonal FLC detectable by MALDI-TOF MS. Most patients with an abnormal serum FLC ratio at this time point, had an abnormal ratio with an increased level of the iFLC (Figure 4.15B). Overall, agreement between serum FLC and FLC-MS testing results was observed in in 38/58 (65.5%) patients (Figure 4.15A).

Of the patients with conflicting FLC-MS and serum FLC ratio results, 9/20 (45.0%) were FLC-MS positive but had a normal serum FLC ratio and 11/20 (55.0%) had no residual detectable monoclonal FLC by MALDI-TOF MS but had an abnormal serum FLC ratio. MRD results were available for 6/11 (54.5%) patients with an abnormal serum FLC ratio but no detectable residual monoclonal FLC by MALDI-TOF MS and all six patients were MRD negative.

3/14 (21.4%) of the patients with an abnormal serum FLC ratio had a GFR <60 ml/minute/1.73m^2 and if the modified renal reference ranges proposed by Long *et al* (Long *et al*., 2022) were applied, all of these patients would be classified as having normal serum FLC ratios. There was no residual monoclonal FLC by MALDI-TOF MS in all three of these patients.

Amongst IFE negative patients with an abnormal serum FLC ratio, kappa light chain patients were again over-represented; kappa was the involved light chain isotype in 14/14 (100%) samples from patients with a persistently abnormal serum FLC ratio.

IFE negative patients with residual detectable monoclonal FLC by MALDI-TOF MS had shorter PFS compared to MS negative patients (Figure 4.15). However, an abnormal serum FLC ratio did not have any statistically significant impact on PFS (Figure 4.15D-F).

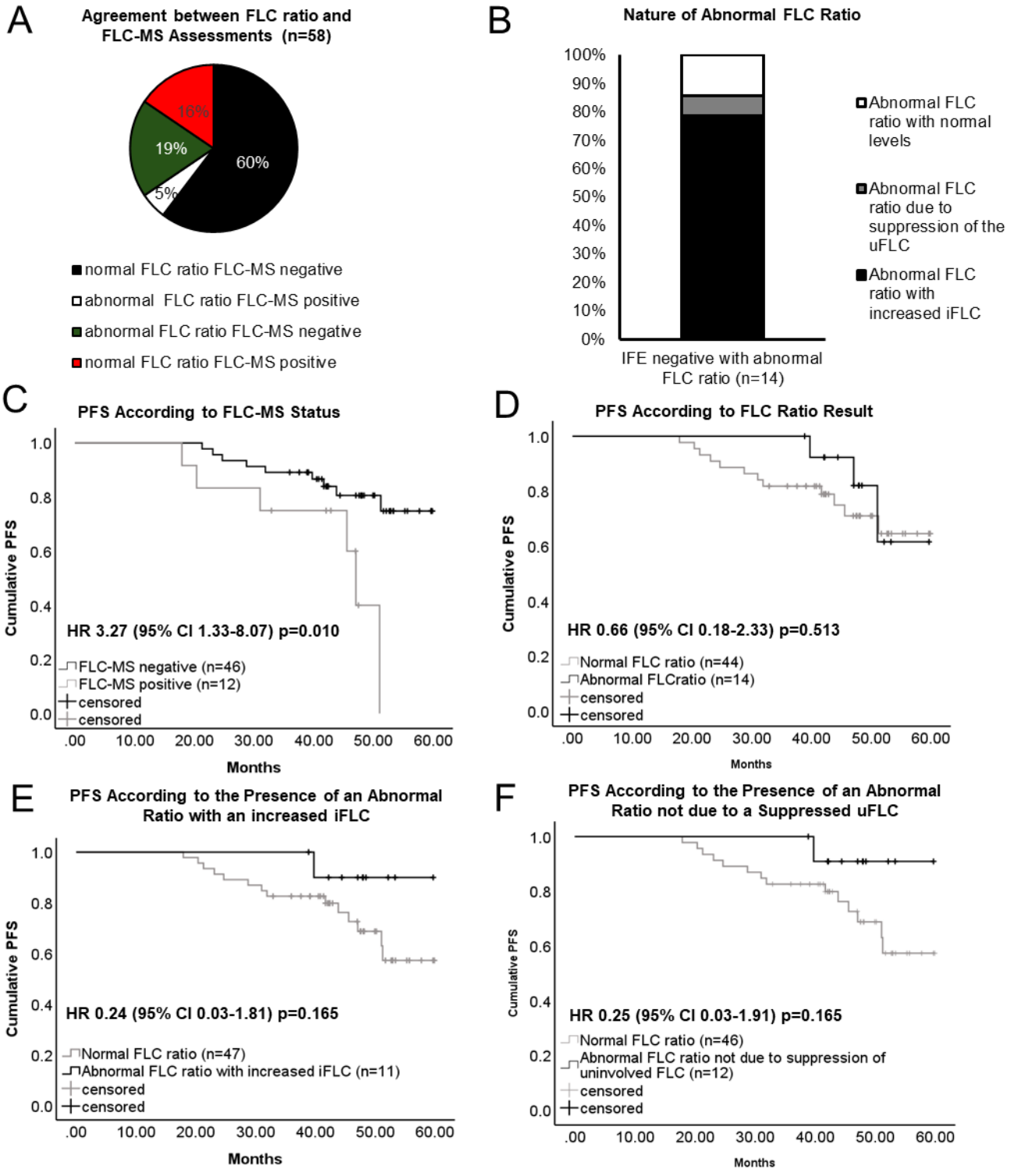


Figure 4. 15 Agreement between serum FLC and FLC-MS assessments in patients treated in the KCRD arm of the Myeloma XI trial who had no detectable monoclonal protein by IFE at six months post maintenance randomisation and their prognostic significance.

(A) Shows the agreement between FLC-MS and serum FLC assessments and (B) shows the nature of the abnormal serum FLC ratio. The PFS of patients according to FLC-MS and serum FLC ratio are shown in (C-F).

4.3.11 Prognostic significance of MALDI-TOF MS status at 15 Months Post ASCT in Patients Treated in the KCRD Arm of the Myeloma XI Trial

107 patients had paired samples from day+100 post ASCT and 15 months post ASCT available for MALDI-TOF MS testing. Overall 75/107 (70.1%) had no detectable residual monoclonal protein by MALDI-TOF MS at 15 months post ASCT and 24/75 (32.0%) of these patients had converted from MALDI-TOF MS positive at day+100 post ASCT. Amongst the patients who were MALDI-TOF MS negative at 15 months post ASCT, a higher proportion of IgG patients had converted from MS positivity at day+100 post ASCT (47.5% v. 14.2%, p=0.005; Figure 4.16A). However, the overall rate of MS negativity did not significantly differ between patients with IgG versus non-IgG monoclonal proteins at 15 months post ASCT (66.7% v. 74.5%, p=0.508; Figure 4.16B). .

There was no difference in the PFS between patients with sustained MALDI-TOF MS negativity versus patients who converted from positive negative between day+100 post ASCT and 15 months post ASCT (Figure 4.16C). However, patients with sustained MS negativity had superior PFS compared to patients who converted from MS negative at day+100 post ASCT to MS positive at 15 months post ASCT (HR 0.08 (95% CI 0.03-0.26), p<0.001) and PFS was similar for patients who patients who converted to MS positivity versus those who never achieved MS negativity.

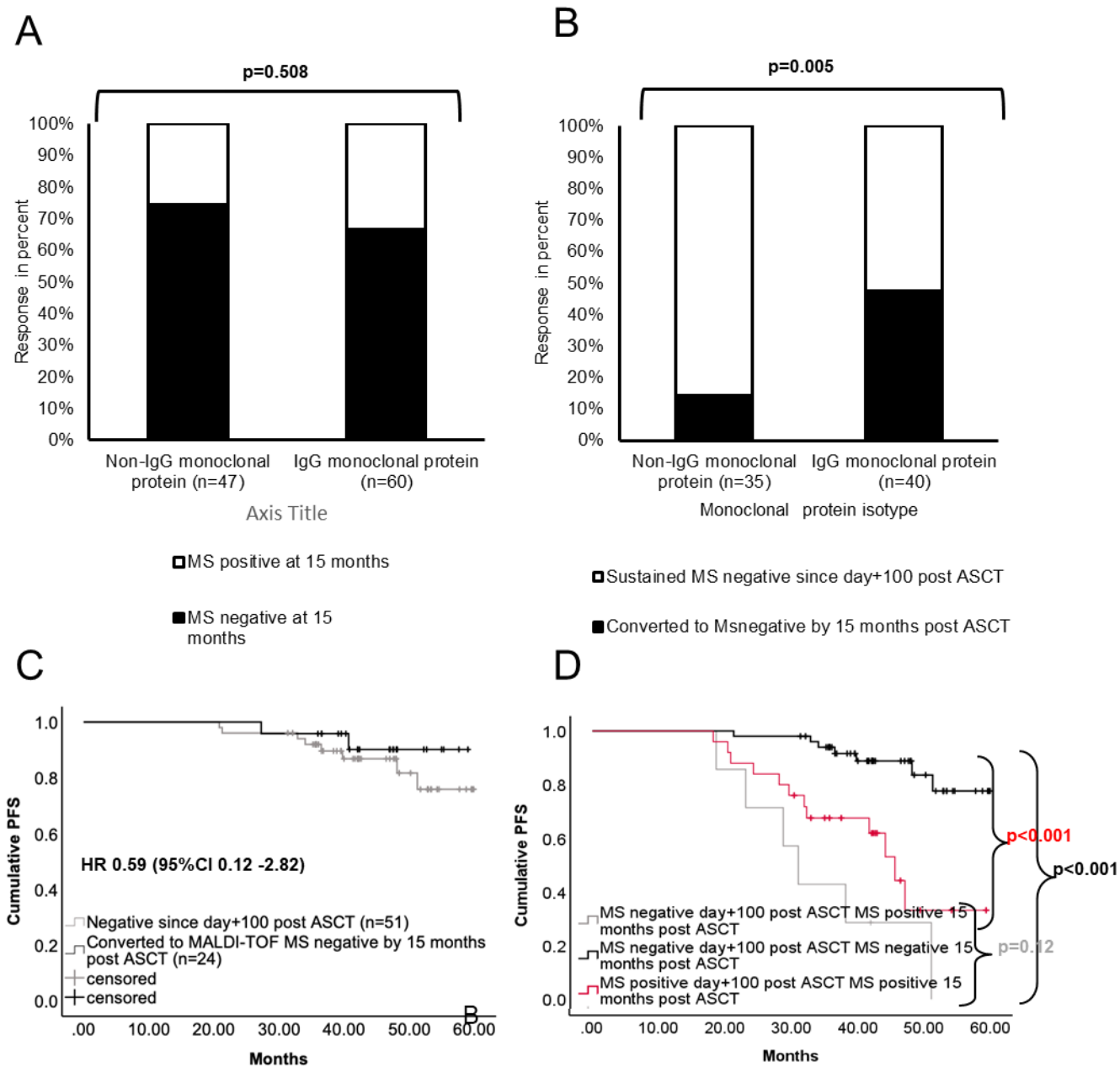


Figure 4. 16 Residual monoclonal protein detection by MALDI-TOF MS at 15 months post ASCT and its prognostic significance

(A) Shows the rate of negativity by MALDI-TOF MS for patients with IgG and non-IgG monoclonal proteins and (B) shows the proportion of patients with sustained MS negativity versus conversion to MS negativity between day+100 post ASCT and 15 months post ASCT. (C-D) Kaplan Meier curves showing the PFS for patients sustained negativity versus those that convert to MS negative re shown in (C) and those that lose MS negativity or never achieved MS negativity are shown in (D).

4.4. Discussion

Overall good agreement between the monoclonal protein isotype identified by the electrophoretic and MALDI-TOF MS assays was observed. The reason for the

infrequent discrepancies is unclear but the rate observed in this work is similar to that reported in previous studies (Mills *et al*., 2016, Puig *et al*., 2022). Although overall agreement for monoclonal protein quantitation assessed by ICC was very good between MS and SPE for patients with intact immunoglobulin monoclonal proteins, there was a trend towards higher monoclonal protein quantitation by MS for high level monoclonal proteins.

From this work it cannot be established how many of the discrepant cases were due to overestimation of the monoclonal protein size by MS due to overestimation of the total immunoglobulin levels used for quantitation and how many may have been due to underestimation by SPE due to dye saturation (Murray *et al*., 2009). Another limitation of the work presented in this chapter is it that it did not include any patients with IgM monoclonal proteins and overestimation of immunoglobulin levels by immunochemical assays in the presence of large monoclonal proteins has been reported to be most problematic in samples containing IgM monoclonal proteins (Murray *et al*., 2009). The linearity data presented by The Binding Site Ltd for the QIP-MS/EXENT assay reported good linearity for monoclonal proteins of 0.014 -88.9 g/L, 0.011-68.4 g/L and 0.11-74.2 g/L for IgG, IgA and IgM monoclonal proteins respectively (Stanton *et al*., 2023), however these results need to be externally validated. This external validation work should include different antisera formulations and batches as these factors have also been shown to influence any bias observed for total immunoglobulin measurements in the presence of monoclonal proteins (Riches *et al*., 1991).

IgE monoclonal proteins are extremely rare (Pandey and Kyle, 2013) and IgD monoclonal proteins are only found in 1-2% of patients with multiple myeloma (Pandey and Kyle, 2013, Agbuduwe et al., 2022), so reagents for these monoclonal protein isotypes have not been included in the initial panel for the QIP-MS/EXENT assay. The three patients with IgD multiple myeloma included in this work therefore had to be monitored by MS like FLC only myeloma patients. Reagents for measuring IgD and IgE by MS need to be developed to maximise applicability of the MS assays and also to permit the correct identification of the overall monoclonal protein isotype by MS.

The results of this study show that MALDI-TOF MS provides greater sensitivity for the detection of residual monoclonal protein compared to electrophoretic assays in patients undergoing systemic treatment for multiple myeloma. The sensitivity of MS was enhanced by using FLC-MS in combination with the QIP-MS/EXENT assay and this increased sensitivity was not limited to patients with FLC only myeloma. In keeping with the greater sensitivity of the MALDI-TOF MS assays relative to SPE and IFE, the rate of MS CR was lower at all time points.

Discordant results between serum FLC and FLC-MS assessments were observed in a significant proportion of patients with discrepancies due to both FLC-MS positivity in patients with normal serum FLC ratios and FLC-MS negativity in patients with abnormal serum FLC ratios. In keeping with several recent studies (Martínez-López *et al*., 2015, Cedena *et al*., 2020), serum FLC ratio normalisation in IFE negative patients did not identify patients with improved PFS. However, FLC-MS negativity at the end of induction, day+100 post ASCT and six months post maintenance

randomisation was associated with a statistically significant improvement in PFS. The negative impact of FLC-MS positivity on PFS at the end of induction and day+100 post ASCT was retained on multivariate analysis. Owing to the limited number of patients and limited number of progression events multivariate analysis was not performed for the six months post maintenance time point in this study.

The results of this study suggest that the lack of prognostic benefit of FLC ratio normalisation in IFE negative patients may have been due both to false positive results and the limited sensitivity of serum FLC assays to detect very low level residual monoclonal FLC.

Due to the greater sensitivity of the MALDI-TOF MS assays compared to electrophoretic techniques it would be expected that it would take longer to achieve MS negativity, particularly for patients with IgG monoclonal proteins as these have the longest half-life. In keeping with this, a higher rate of conversion to MS negativity between day+100 post ASCT and 15 months post ASCT was observed in patients with IgG secreting disease compared to non-IgG secreting disease but by 15 months post ASCT MS negativity rates were similar between patients with IgG monoclonal protein versus those with non-IgG monoclonal proteins. The impact of the long half-life of IgG monoclonal proteins may also at least in part explain why the agreement between MS and bone marrow MRD was 75% at the six month post maintenance randomisation time point compared to only 58% at the end of induction chemotherapy.

Further studies are needed to understand the kinetics of response assessments using MALDI-TOF MS and identify the optimal time points for testing, particularly given their higher cost in comparison to the more widely available electrophoretic assays (Giles *et al*., 2022). Based on the results of this work these response kinetics studies should focus on the time period between 3 and 15 months post ASCT. Additional studies evaluating the response kinetics by MS in non-transplant eligible patients should also be undertaken. These studies may also be crucial as response adapted maintenance strategies are being explored in several ongoing studies (Royle *et al*., 2022, Derman *et al*., 2022) and MS assays may be explored in this context in future studies..

Although the analysis of the sensitivity and prognostic utility of MS-based response assessments presented in this chapter are encouraging, the work does have some limitations. Firstly, the sensitivity of the flow cytometry-based MRD was low compared to more modern panels such as NGF and MRD results were not available in a significant number of the patients.

Secondly, no follow-up imaging studies were incorporated into the trial. It is therefore not possible to ascertain how many of the discrepant results between MALDI-TOF MS and MRD were due to low-level residual disease not involving the posterior iliac crest and how many may have been due to the limited sensitivity of the MRD assay used in this trial. Spatial and genomic heterogeneity is a common feature in multiple myeloma (Mithraprabhu *et al*., 2017, Rasche *et al*., 2017b) and patients may present with diffuse marrow involvement or multiple focal lesions (Messiou *et al*., 2021). In keeping with the patchy nature of myeloma infiltration seen in some patients, rates of residual

disease detectable by functional imaging of up to 20% have been reported for patients who are negative for MRD in the bone marrow (Alonso *et al*., 2019, Rasche *et al*., 2019, Belotti *et al*., 2021, Böckle *et al*., 2022, Fonseca *et al*., 2023). Follow-up studies evaluating MS-based response assessments in comparison to functional imaging and bone marrow MRD assessments should be conducted to identify how MS-based assays may be used to supplement and/or guide the timing of testing using these other assessments. Follow-up reflex testing with LC-MS, which provides greater resolution and sensitivity than MALDI-TOF MS, may provide further insights into how many patients who were MRD positive but MALDI-TOF MS negative had low-level monoclonal protein below the limit of detection of the QIP-MS/EXENT and FLC-MS assays.

Thirdly, none of the patients included in this study received induction chemotherapy containing an anti-CD38 monoclonal antibody, which would produce deeper responses (Moreau *et al*., 2019, Voorhees *et al*., 2020, Menon *et al*., 2021) and where a greater proportion of patients would therefore be expected to achieve MALDI-TOF MS negativity.

Lastly, the duration of follow-up was too short to enable OS analysis to be performed as only a small proportion patients have died. This analysis should be performed on the samples tested as part of this thesis once sufficient follow-up data is available.

5:THE UTILITY OF MS FOR THE DETECTION AND MONITORING OF MONOCLONAL PROTEINS IN PATIENTS WITH NON-MEASURABLE MULTIPLE MYELOMA

5.1 Introduction

Most patients with multiple myeloma have a detectable monoclonal protein in their serum and/or urine and this provides a biomarker for assessing disease status and response to anti-myeloma treatment. However, in 1-3% of patients with newly diagnosed multiple myeloma there is no detectable monoclonal protein in the serum or urine and these patients are categorised as having NSMM (Drayson M, 2001, Chawla *et al*., 2015, Kumar *et al*., 2016, Heaney *et al*., 2017, Migkou *et al*., 2020). Laboratory studies have identified that the absence of a detectable monoclonal protein may arise due to four separate mechanisms. Firstly, the neoplastic cells may secrete very small amounts of monoclonal protein resulting in a monoclonal protein levels in the serum which are below the lower limit of detection of the current serological assays (oligo-secretors). Secondly, the monoclonal protein may be secreted within vesicles, preventing reagent binding (false non-secretors). Thirdly, the neoplastic cells may have a mutation which affects its ability to secrete the monoclonal protein (non-secretors). Lastly, the neoplastic cells may have a mutation which results in no immunoglobulin production (true non-producers) (Corso and Mangiacavalli, 2017).

In order for the monoclonal protein to be used as a biomarker to assess response, the monoclonal protein needs to present at a sufficient concentration to permit detection of a PR and VGPR. Owing to the limits of sensitivity of the currently utilised electrophoretic techniques and the difficulties in accurately being able to quantitate small monoclonal proteins, the IMWG defines measurable disease using electrophoretic techniques as a serum monoclonal protein concentration of ≥10 g/L and/or urine monoclonal protein ≥200 mg/24 hours. For patients with no measurable monoclonal protein using the standard electrophoretic techniques, the IMWG permits the use of serum FLC measurements providing the iFLC is ≥100 mg/L and the FLC ratio is abnormal (Kumar *et al*., 2016). Using this combination of methodologies 95-97% of patients have measurable disease (Wålinder *et al*., 2020, Migkou *et al*., 2020). Patients presenting with multiple myeloma who have detectable monoclonal protein in the serum or urine but who do not fulfil the IMWG criteria for measurable disease are described as having non-measurable oligo-secretory multiple myeloma.

Serial bone marrow biopsies are the gold standard methodology for assessing response in patients who do not have a measurable monoclonal protein in their serum and/or urine at presentation, providing the plasma cell infiltrate is ≥30% (Kumar *et al*., 2016). However, many newly diagnosed patients with non-measurable multiple myeloma have been reported to have plasma cell infiltration <30% (Wålinder *et al*., 2020, Migkou *et al*., 2020). In addition, a bone marrow biopsy is an invasive and uncomfortable procedure and therefore cannot be performed as frequently as blood and urine assessments. The IMWG recommends that bone marrow biopsies are

performed every 3-4 cycles until a plateau is reached and then at suspected disease progression (Kumar *et al*., 2016). However, this pragmatic approach of reduced testing frequency can lead to delays in the identification of progressive disease and therefore lead to increased morbidity, particularly related to lytic bone lesions.

Bone marrow infiltration is commonly heterogenous in multiple myeloma and there may be areas with only a few neoplastic cells depending on the sampling site (Fujino, 2018). Adequately sized trephine biopsies are required to ensure that poor sample quality does not lead to false negative results. In order to minimise the risk of a false negative results on bone marrow biopsy and to avoid the discomfort of serial bone marrow biopsies, many patients with non-measurable multiple myeloma are monitored with serial PET-CT scans in real-world practice. However, this approach is not universally applicable as approximately 10% of patients have low hexokinase levels, which can cause false negative PET-CT results (Rasche *et al*., 2017a, Abe *et al*., 2019). In addition, serial PET-CT scans are associated with repeated exposure to high doses of ionising radiation, significant cost and access to the specialised imaging equipment is not available at all NHS hospitals (Westerland *et al*., 2021b). MRI scans provide an alternative imaging modality for assessing myelomatous disease burden, however its widespread employment is limited by capacity issues and MRI has not been approved by the IMWG for response assessment (Kumar *et al*., 2016, Westerland *et al*., 2021b).

These challenges in being able to accurately assess response in patients with non-measurable multiple myeloma mean that these patients may undergo excessive

numbers of bone marrow biopsies or cross-sectional imaging procedures. In addition they are frequently excluded from clinical trials (in the first 100 interventional trials for myeloma on clinicaltrials.gov (accessed April 27 2021) no trials specifically include NSMM or oligo-secretory multiple myeloma, 54 exclude NSMM and OSMM, 3 exclude patients with NSMM but will permit patients with oligo-secretory multiple myeloma, 7 will accept patients with measurable disease on PET-CT and 36% did not have sufficient detail to evaluate eligibility for NSMM or oligo-secretory multiple myeloma), limiting their access to novel agents which may improve outcomes.

Immunohistochemical studies have demonstrated evidence of monoclonal protein production is present in 40-85% of patients with NSMM (Hobbs, 1967, Turesson and Grubb, 1978), suggesting that more sensitive methodologies for measuring monoclonal protein production may be able to detect monoclonal protein in a significant proportion of patients who are currently classified as having NSMM.

Mass spectrometry assays have emerged as a new methodology for monitoring monoclonal protein levels in the peripheral blood and preliminary results have suggested that they are able to offer greater sensitivity than the currently utilised electrophoretic techniques (Ashby JA, 2018). They have also been demonstrated to be able to detect low-level monoclonal proteins in a greater proportion of samples from patients who subsequently went on to develop MGUS (Murray *et al*., 2019) and have been shown to provide prognostically relevant information in patients undergoing treatment for multiple myeloma and AL amyloidosis (Dispenzieri *et al*., 2020a,

Dispenzieri *et al*., 2022, Puig *et al*., 2022). However, to date their potential utility in patients with non-measurable myeloma has not been studied.

Discrepancies between the monoclonal protein isotypes reported by MALDI-TOF MS versus IFE have been observed in 2-3% of patients in previously reported MALDI-TOF MS studies (Mills *et al*., 2016, Puig *et al*., 2022). However, the histological results were not reported in either of these studies, so it is unclear which technology detected the correct monoclonal protein isotype.

The aims of the work included in this chapter were to:

- Evaluate whether there was agreement between the low-level monoclonal proteins detected by MALDI-TOF MS and the histology results in patients presenting to UHB with no detectable monoclonal protein by IFE at presentation and/or first relapse of a PCD. This was done to ensure that oligoclonal peaks of a different specificity were not being identified as monoclonal protein peaks in MS spectra obtained from patients with PCD with low-level monoclonal protein production
- To assess the detection rate of monoclonal proteins in patients with NSMM and whether these low-level monoclonal proteins could be tracked through the course of treatment
- To evaluate whether LC-MS may provide greater sensitivity for the monitoring of patients with monoclonal proteins close to the limit of sensitivity for the QIP-MS/EXENT assay

- To assess the agreement between the monoclonal proteins detected by MALDI-TOF MS and electrophoretic techniques in patients with oligo-secretory myeloma who were treated in the Myeloma XI trial monoclonal protein and to evaluate whether MALDI-TOF MS could provide greater sensitivity for monitoring these patients.
- To evaluate whether MALDI-TOF MS could be used to detect a VGPR in patients with non-measurable myeloma.

5.2. Methods

5.2.1 Assessment of Patients with Non-measurable PCD from UHB NHS Foundation Trust

Serum samples were collected from consenting patients undergoing treatment for symptomatic PCD at UHB NHS Foundation Trust in whom there was no detectable monoclonal protein by IFE at presentation and/or first relapse. QIP-MS/EXENT and FLC-MS testing was performed on samples from presentation and/or first relapse. The spectra were manually reviewed for the presence of a monoclonal protein blinded to the histology results. The results of the MS testing were compared to the histology results to assess the agreement between the monoclonal proteins identified by MS and the immunoglobulin expression pattern on the neoplastic plasma cells.

5.2.2 Assessment of Samples from Patients with Non-measurable Multiple Myeloma from the Myeloma XI Trial

Patients treated in the Myeloma XI trial whose monoclonal protein and serum FLC results did not fulfil the IMWG criteria for measurable disease (monoclonal protein <10 g/L, BJP <200 mg/24 hours and iFLC <100 mg/L) were included in this study. All samples underwent MALDI-TOF-MS and FLC-MS testing. Due to the low volume of

many of the presentation samples and shortages in tips for the automated liquid handler due supply issues during the COVID-19 pandemic, the immune enrichment steps of the QIP-MS/EXENT and FLC-MS assays were performed manually for many of these samples. The eluates were spotted onto the target plates using the automated liquid handler as described in Section 2.6. The spectra were manually reviewed for the presence of a monoclonal protein blinded to the results of the central laboratory testing. Quantitative analysis of the intact immunoglobulin monoclonal protein peaks was performed retrospectively once a prototype version of the software had been developed.

Figure 5.1 is a CONSORT diagram showing the patients from the Myeloma XI trial included in this study. Patients that were excluded comprised: (i) patients with measurable disease using SPE, BJP and/or serum FLC assessment; and (ii) patients with insufficient residual serum from their baseline sample for MALDI-TOF MS testing.

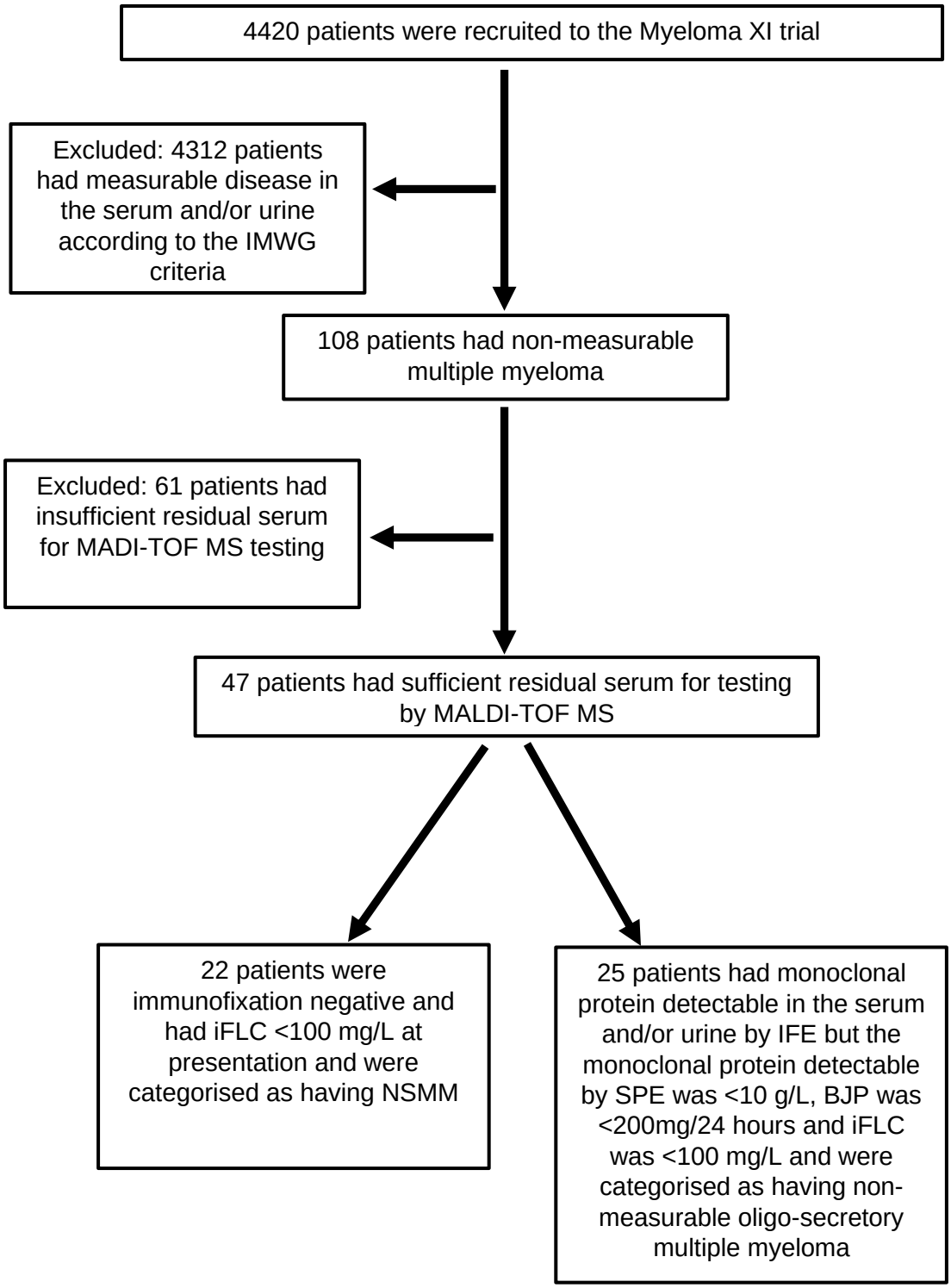


Figure 5. 1 CONSORT Diagram showing the serum samples analysed by MALDI-TOF MS from the Myeloma XI trial

Patients with non-measurable myeloma were divided into two cohorts; patients with NSMM and patients with oligo-secretory myeloma. All available serum samples between presentation and day +100 post ASCT were included in this study and underwent testing with QIP-MS/EXENT and FLC-MS.

In samples in which a low-level intact immunoglobulin monoclonal protein close the limit of detection of the QIP-MS/EXENT assay was detected, LC-MS testing was also performed to evaluate how many additional samples had residual disease detectable using this more sensitive MS methodology.

5.3. Results

5.3.1 Agreement between MALDI-TOF MS results and Histological Results in Five Patients Diagnosed with Symptomatic PCD at UHB NHS Foundation Trust in whom there was no Detectable Monoclonal Protein by IFE

In this study agreement between the monoclonal protein identified by MALDI-TOF MS and the immunohistochemistry results was assessed in five patients with symptomatic presentation of a PCD being treated at University Hospitals Birmingham NHS Foundation Trust in whom there was no detectable monoclonal protein by SPE or IFE. 4/5 (80.0%) of patients had multiple myeloma and 1/5 (20.0%) had a solitary plasmacytoma. The details of the samples included in this study are listed in Table 5.1.

Monoclonal protein was identified by MALDI-TOF MS in the serum of 3/5 (60.0%) of the samples. There was 100% agreement between the monoclonal light chain identified by MALDI-TOF MS and the light chain expression observed on immunohistochemical analysis of the biopsy. 2/5 (40.0%) patients had an abnormal serum FLC ratio but neither had measurable disease by Freelite® and 3/5 (60.0%) of samples had monoclonal FLC detectable by MALDI-TOF MS.

1/5 (20%) patients had an intact immunoglobulin monoclonal protein identified by MALDI-TOF MS, which had the same *m/z* as the monoclonal kappa FLC identified by MALDI-TOF MS. No IgG heavy chain expression was observed on the neoplastic plasma cells identified in the scapula biopsy and the bone marrow biopsy contained no evidence of infiltration by neoplastic plasma cells. The patient has been treated with radiotherapy for a solitary plasmacytoma and currently remains in clinical remission with no detectable monoclonal protein and a normal serum FLC ratio. Longer term follow-up is needed to see if this patient will progress to symptomatic multiple myeloma and if so, whether any of the neoplastic plasma cells show evidence of IgG production.

Table 5. 1 Histological, serological and MALDI-TOF MS results from patients being treated at UHB NHS Foundation Trust who presented with no detectable monoclonal protein on IFE at initial diagnosis and/or relapse

Sample ID	Diagnosis	Stage	Histology	Electrophoresis	sFLC (mg/L)	eGFR	MALDI-TOF MS
UHB 1	Multiple myeloma	Presentation	Heavy plasma cell infiltrate. No light chain expression.	Decreased immunoglobulins	sFK11.63 sFL 11.55	>90	Negative
UHB 2	Multiple myeloma	Presentation	Neoplastic plasma cell infiltrate. No light chain expression.	Decreased immunoglobulins	sFK 1.96 sFL 1.94	78	Negative
UHB 3	Solitary plasmacytoma	Presentation	Kappa restricted plasma cells. Negative for IgG, IgA, IgM and IgD.	Polyclonally increased immunoglobulins	sFK 20.29 sFL 16.13	>90	IgG kappa monoclonal protein *m/z* 11860 and monoclonal kappa FLC *m/z* 11861
UHB 4	Multiple myeloma	1st relapse	Kappa restricted plasma cells.	Decreased immunoglobulins	sFK 25.35 sFL 3.45	>90	Glycosylated monoclonal kappa FLC
UHB 5	Multiple myeloma	Presentation	Kappa restricted plasma cells. Negative for IgG, IgA, IgM and IgD.	Decreased immunoglobulins	sFK 39.52 sFL 10.16	9	Glycosylated monoclonal kappa FLC

5.3.2 Baseline Characteristics of the Patients with Non-Measurable Multiple Myeloma in the Myeloma XI Trial

47 patients whose presentation monoclonal protein and serum FLC levels did not fulfil the IMWG criteria for measurable disease had sufficient residual serum for testing by MALDI-TOF MS. 22/47 (46.8%) patients had no detectable monoclonal protein by IFE and had an iFLC <100 mg/L and were categorised as having NSMM. 25/47 (53.2%) patients had monoclonal protein detectable by serum IFE/SPE and/or urine IFE but the level was below the minimum levels specified by the IMWG for measurable disease and these patients were described as having non-measurable oligo-secretory multiple myeloma.

Table 5.2 summarises the baseline characteristics of the patients included in this study. None of the patients presented with severe renal impairment; the median creatinine level at presentation was 85 μmol/L (range 35-152 μmol/L). ISS results were available for 46/47 (97.9%) patients and overall, 33/47 (70.2%) had ISS stage 1 disease and only 4/47 (8.5%) had ISS stage 3 disease at presentation. 38/47 (80.9%) of patients had immune paresis at presentation.

Table 5. 2 Baseline laboratory parameters for the patients who presented with non-measurable multiple myeloma

Clinical Parameter	Number of Patients (%)
NSMM (n=22)	
Immunofixation positive	0 (100)
Normal FLC ratio	8 (36.4)
iFLC <50 mg/L with abnormal FLC ratio	7 (31.8)
iFLC 50-100mg/L with abnormal FLC ratio	7 (31.8)
Immune paresis	17 (77.3)
Serum creatinine <177 μmol/L	22 (100.0)
ISS 1	16 (72.7)
ISS 2	4 (18.2)
ISS 3	2 (9.1)
Oligo-secretory myeloma (n=25)	
Immunofixation positive	25 (100.0)
IgG monoclonal protein	13 (52.0)
IgA monoclonal protein	10 (40.0)
Light chain only	1 (4.0)
Biclonal	1 (4.0)
Monoclonal protein <5g/L (range 0 g/L-4.11 g/L)	8 (32.0)
Monoclonal protein 5-<10 g/L (range 5.0 g/L-9.3 g/L)	17 (68.0)
Normal FLC ratio	4 (16.0)
iFLC <50mg/L with abnormal FLC ratio	17 (68.0)
iFLC 50-100mg/L with abnormal FLC ratio	4 (16.0)
Urinary light chains <200 mg/g creatinine (range 0-110 mg/g creatinine)	23 (92.0)
Missing urinary light chains results	2 (8.0)
Immune paresis	21 (84.0)
Serum creatinine <177 μmol/L	25 (100.0)
ISS 1	17 (68.0)
ISS 2	6 (24.0)
ISS 3	1 (4.0)
ISS missing	1 (4.0)

5.3.3. Monoclonal Protein is Detectable in Most Patients treated in the Myeloma XI Trial who Presented with NSMM

Overall, monoclonal protein was detectable by MALDI-TOF MS in 20/22 (90.9%) baseline samples from patients with NSMM. 7/22 (31.8%) of patients with NSMM had an intact immunoglobulin monoclonal protein but no monoclonal FLC detectable by MALDI-TOF MS in their baseline samples. In 5/7 (71.4%) patients the monoclonal protein was present at a sufficient level to be quantified by MALDI-TOF MS (range 9-260 mg/L) (example spectra shown in Figure 5.2). 4/7 (51.7%) of patients had an IgG monoclonal protein, 2/7 (28.6%) had an IgA monoclonal protein and 1/7 had biclonal disease (one IgG monoclonal protein and one IgA monoclonal protein). 3/7 (42.9%) patients had an abnormal serum FLC ratio at presentation and in all of these patients the iFLC was <50 mg/L (range 25.8 – 46.6 mg/L). In 2/3 (66.7%) patients with an abnormal FLC ratio the light chain isotype of the intact immunoglobulin monoclonal protein identified by MALDI-TOF MS matched the direction of skew of the FLC ratio.

6/22 (27.3%) of patients with NSMM had detectable monoclonal FLC and intact immunoglobulin monoclonal protein. In 3/6 (50.0%) patients the monoclonal FLC had the same *m/z* and were of the same isotype as the light chain component of the intact immunoglobulin monoclonal protein, indicating that they have arisen from the same neoplastic plasma cell population. The intact immunoglobulin monoclonal protein was quantifiable by MALDI-TOF MS in 3/3 (100%) of these patients (range 86-140 mg/L). The serum FLC ratio was only abnormal in 1/3 (33.3%) of these patients.

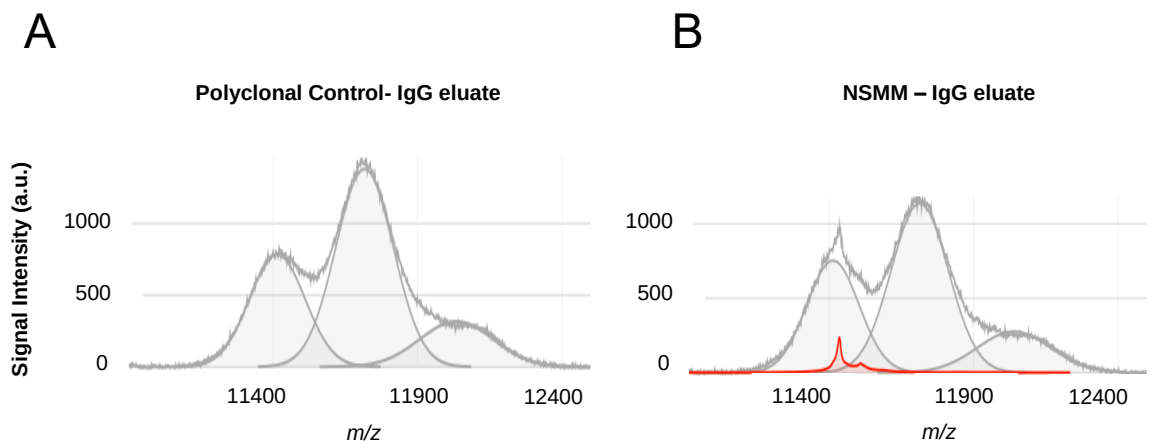


Figure 5. 2 MALDI-TOF MS is able to identify quantifiable intact immunoglobulin monoclonal proteins in pateints in patients with NSMM

An example of an IgG spectrum from polyclonal serum from healthy blood donors analysed using the EXENTiQ software is shown in (A). (B) shows the mass spectrum obtained from the IgG eluate of a NSMM sample in which a 140 mg/L IgG lambda monoclonal protein was identified by MALDI-TOF MS. The grey area represents the total area for IgG and the red line and shaded area represents the modelled area for the monoclonal protein identified and quantified by the EXENTiQ software (system and assay under development).

7/22 (31.8%) of patients with NSMM had FLC only monoclonal proteins detectable by MALDI-TOF MS. In 6/7 (85.7%) of patients the iFLC was kappa and in the remaining 1/7 (14.3%) the iFLC was lambda. 7/7 (100%) of these patients had an abnormal FLC ratio at diagnosis. In 6/7 (85.7%) patients the isotype of the monoclonal FLC identified by MALDI-TOF MS was the same as indicated by the skewed serum FLC ratio (iFLC 16.3-90.3 mg/L), including 4/4 (100%) of patients where the iFLC was >50 mg/L. In the sample where a discrepancy between Freelite® and MALDI-TOF MS results was observed, the serum FLC ratio was only just outside the normal range at 1.72 in the context of a moderately reduced eGFR of 58 ml/minute. This ratio would be classified as normal using the recently proposed modified reference ranges for patients with chronic kidney disease based on the screening results from the iSTOPMM study (Long *et al*., 2022).

Both of the patients in whom there was no detectable monoclonal protein detectable by MALDI-TOF MS at baseline, presented with immune paresis, normal serum FLC levels and had normal serum FLC ratios.

5.3.4 MALDI-TOF MS Can Provide a Useful Adjunct for Monitoring Patients with NSMM

39 follow-up samples were available for MALDI-TOF MS testing from patients treated for NSMM. The details of the timepoints and results of the electrophoretic, Freelite® and MALDI-TOF MS testing results are listed in Table 5.3.

Residual monoclonal protein was detectable by MALDI-TOF MS in 11/15 (73.3%) samples from the end of the first cycle of induction chemotherapy. In 4/11 (36.4%) samples the residual monoclonal protein was only detectable by FLC-MS. In only 1/4 (25.0%) of these patients was the dFLC ≥10 mg/L but the serum FLC ratio was abnormal in 3/4 (75.0%) of these patients (range 0.80 – 7.00).

Post-induction, 1/11 samples (9.1%) had an abnormal sFLC ratio. Residual monoclonal FLC were detectable by MALDI-TOF MS in 3/11 (27.3%) samples, including the samples with a persistent abnormal serum FLC ratio. In all three samples the residual FLC only monoclonal protein was only detectable by FLC-MS. Residual intact immunoglobulin monoclonal protein was also detectable by MALDI-TOF MS in 2/11 (18.2%) of samples.

2/5 (40.0%) samples from day+42 post ASCT and 2/8 (25.0%) samples from day+100 post ASCT had residual monoclonal protein detectable by MALDI-TOF MS. The residual monoclonal protein was detectable by EXENT/QIP-MS in 2/4 (50.0%) patients

and in 2/4 (50.0%) the residual monoclonal FLC was only detectable by FLC-MS. Both of the samples with residual monoclonal FLC detectable by MALDI-TOF MS had normal serum FLC ratios and the dFLC was <10mg/L. 1/13 (7.7%) of samples from post-ASCT had an abnormal serum FLC ratio in the context of suppressed FLC levels (serum free kappa 5.35 mg/L, serum free lambda 1.56 mg/L, sFLC ratio 3.43). No residual monoclonal FLC were detectable by MALDI-TOF MS in this sample, suggesting that the residual abnormal FLC ratio may have been due to treatment related immune suppression rather than persistent disease.

Table 5. 3 Rates of detection of residual monoclonal protein using electrophoretic assays, Freelite® and MALDI-TOF MS in patients presenting with NSMM

	Residual Monoclonal Protein Detectable by SPE and/or IFE, n (%)	Monoclonal Protein Detectable by MALDI-TOF MS, n (%)	Samples with an Abnormal FLC ratio, n (%)	Samples with Elevated iFLC and Abnormal FLC ratio, n (%)
Post Cycle 1 (n=15)	na	11 (73.3)	9 (60.0)	5 (33.3)
Post Induction (n=11)	na	5 (45.5)	1 (9.1)	0 (0)
Day+42 Post ASCT (n=5)	na	2 (40.0)	0 (0)	0 (0)
Day+100 Post ASCT (n=8)	na	2 (25)	1 (12.5)	0 (0)

Two samples from patients with NSMM were diluted into serum from healthy blood donors mixed with PBST (IgG 4.7 g/L, IgA 1.0 g/L, IgM 0.49 g/L, serum free kappa 4.99 mg/L and serum free lambda 7.35 mg/L) and analysed by MALDI-TOF MS to assess whether MALDI-TOF MS may have potential utility for response assessment in patients with NSMM. In the sample from a patient with iFLC 48 mg/L at diagnosis, residual monoclonal FLC was still detectable following a 1:20 dilution. However, there

was no detectable residual monoclonal protein by MALDI-TOF MS following a 1:2 dilution of a sample from a patient who had 10 mg/L biclonal IgA monoclonal proteins at diagnosis. These results suggest that whilst the additional sensitivity provided by MALDI-TOF MS may mean that some patients can be monitored using this technique in other patients it may only serve as a marker of disease activity to signpost clinicians to perform further staging investigations as it is not detectable at a sufficient concentration to be used for response assessment.

5.3.5 LC-MS provides greater sensitivity for the detection of residual intact immunoglobulin monoclonal proteins compared to MALDI-TOF MS

LC-MS analysis was performed on 17 follow-up samples from eight patients with low-level intact immunoglobulin monoclonal proteins that were close to the limits of detection by MALDI-TOF MS at presentation to determine how many had residual monoclonal protein detectable by LC-MS. The results of this testing are listed in Table 5.4. Residual monoclonal protein was detectable in 13/17 (76.5%) of these samples by LC-MS (example mass spectra are shown in Figure 5.3), including four samples in which there was no residual monoclonal protein detectable by MALDI-TOF MS.

Table 5. 4 Rates of detection of residual monoclonal protein by MALDI-TOF MS and LC-MS in Patients with NSMM who had low-level Intact Immunoglobulin Monoclonal Proteins Detectable by MALDI-TOF MS

	Negative by Serum Protein Electrophoresis and Immunofixation (n=17)
Percentage of samples positive by MALDI-TOF MS and LC-MS (n)	52.9 (9)
Percentage of samples negative by MALDI-TOF-MS but positive LC-MS (n)	23.5 (4)
Percentage of samples positive by MALDI-TOF MS and negative by LC-MS (n)	0 (0)
Percentage of samples negative by MALDI-TOF MS and LC-MS (n)	23.5 (4)

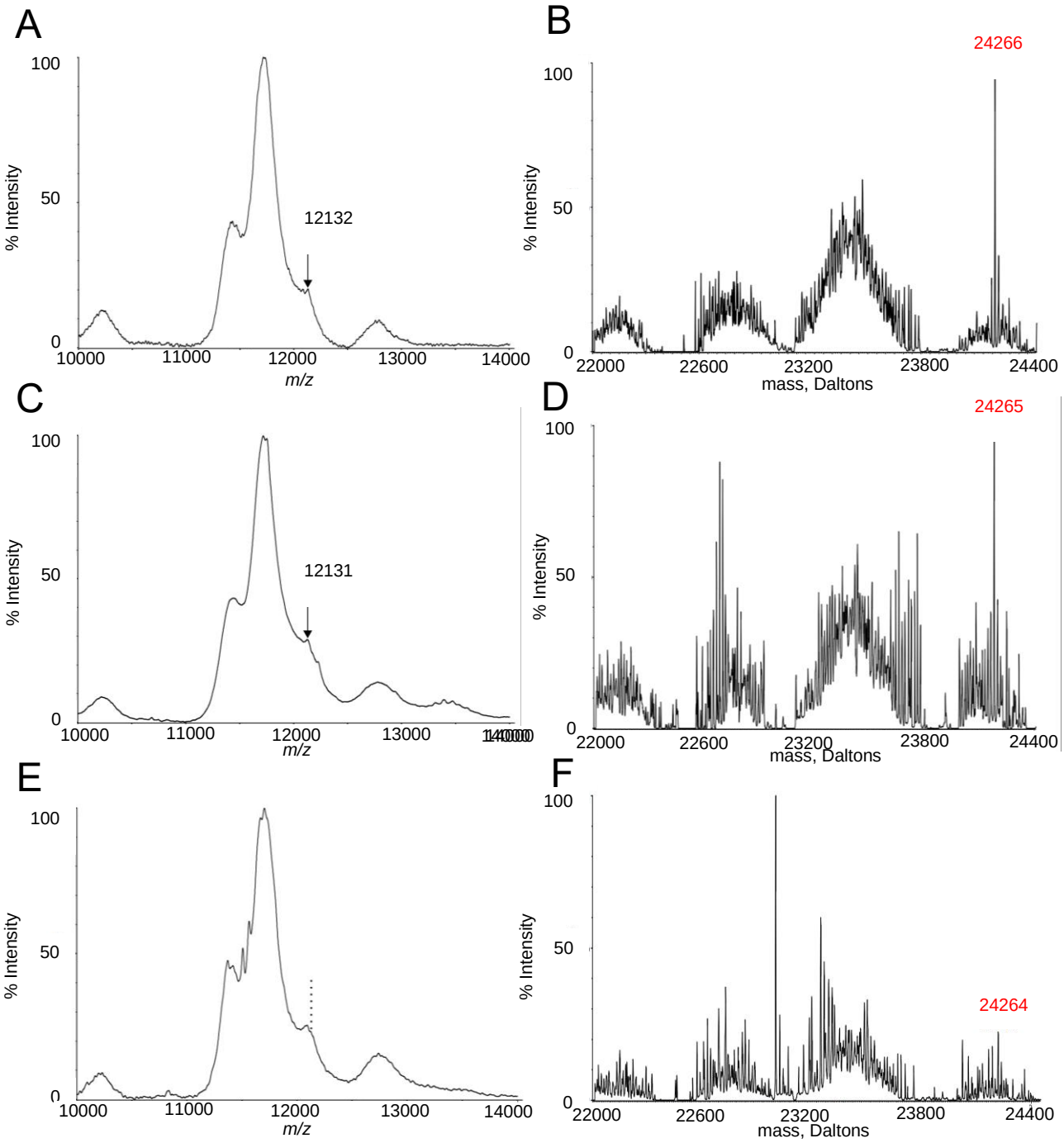


Figure 5. 3 LC-MS provides added sensitivity for the detection of low-level monoclonal production in patients with NSMM

A small IgG kappa monoclonal protein at m/z 12132 for the doubly charged light chain was detectable by MALDI-TOF MS at baseline (A) and post cycle one of induction chemotherapy (C) (highlighted by the black arrows). Post-ASCT oligoclonal peaks are present at m/z 11367, 11506, 11567 and 12090 for the doubly charged light chain but there was no residual measurable IgG kappa monoclonal protein at m/z 12132 (m/z 12132 is highlighted by the dashed line). LC-MS detected an IgG kappa monoclonal protein at 24060 Daltons at baseline (B) and residual monoclonal protein with the same mass was detectable at the end of cycle one of induction chemotherapy (highlighted by the red mass label in D) and post-ASCT (highlighted by the red mass label in F).

Efforts to test samples from patients with FLC secreting disease by LC-MS were unsuccessful due to interference from the high levels of polyethylene glycol in the eluates. Further development work exploring whether guard columns and/or substituting TWEEN 20 for an LC-MS compatible detergent needs to be undertaken to optimise the assay for monoclonal FLC detection.

5.3.6 Monoclonal Protein Detection Using MALDI-TOF MS in Patients with Non-measurable Oligo-secretory Multiple Myeloma treated in the Myeloma XI trial

24/25 (96.0%) patients with oligo-secretory multiple myeloma had detectable intact immunoglobulin monoclonal protein production detectable by serum IFE; 13/25 (52.0%) patients had a single IgG monoclonal protein; 10/25 (40.%) had a single IgA monoclonal protein; and 1/25 (4.0%) had an IgA and an IgG monoclonal protein detectable by immunofixation. 1/25 (4.0%) patients had a monoclonal lambda light chains detectable by urine IFE with no detectable intact immunoglobulin monoclonal protein. Monoclonal protein of the same isotype as detected by IFE was detectable by MALDI-TOF MS in all serum samples and a second IgA monoclonal protein was detectable by MALDI-TOF MS in a patient with a single IgA monoclonal protein detectable by serum IFE. 5/25 (20.0%) of the patients had mass spectra consistent with N-linked glycosylation of the monoclonal light chain.

5.3.7 MALDI-TOF MS Detected Residual Monoclonal Protein in a Greater Proportion of Patients with Oligo-secretory Multiple Myeloma Compared to IFE

The results of central laboratory analysis testing and MALDI-TOF MS are summarised in Table 5.5. 19/25 (76.0%) patients had sufficient residual serum available for MALDI-TOF MS testing from post cycle one of induction chemotherapy. At the end of the first

cycle of induction chemotherapy residual monoclonal protein was detectable using the standard electrophoretic techniques in 18/19 (94.7%) samples and 19/19 (100%) samples by MALDI-TOF MS. The sample with no detectable residual monoclonal protein by IFE was from the patient with a lambda FLC only monoclonal protein at presentation and the serum FLC values and sFLC ratio were normal (serum free kappa 5.74 mg/L, serum free lambda 12.11 mg/L, sFLC ratio 0.474).

Table 5. 5 Monoclonal protein detection rates by electrophoresis, serum FLC assessment and MALDI-TOF MS in patients with oligo-secretory multiple myeloma

	Residual Monoclonal Protein Detectable by SPE and/or IFE, n (%)	Monoclonal Protein Detectable by MALDI-TOF MS, n (%)	Abnormal FLC ratio, n (%)	Elevated iFLC and Abnormal FLC ratio, n (%)
Post Cycle 1 (n=19)	18 (94.7)	19 (100)	7 (36.8)	4 (21.1)
Post Induction (n=11)	8 (72.7)	9 (81.8)	5 (45.5)	2 (18.2)
Day+42 Post ASCT (n=4)	3 (75.0)	3 (75.0)	2 (50.0)	0 (0)
Day+100 Post ASCT (n=4)	1 (25.0)	2 (50.0)	2 (50.0)	0 (0)

Residual monoclonal protein was detectable using the standard electrophoretic techniques in 8/11 (72.7%) and 9/11 (82.7%) of samples from the end of induction chemotherapy using the standard electrophoretic techniques and MALDI-TOF MS respectively. In the sample in which no residual monoclonal protein was detectable by SPE and IFE, MALDI-TOF MS detected 0.12 g/L residual IgG kappa monoclonal protein. There were no detectable residual monoclonal FLC in this sample using

Freelite® (serum free kappa 7.89 mg/L, serum free lambda 7.75 mg/L, sFLC ratio 1.018).

Residual monoclonal protein was detectable in 3/4 (75.0%) samples from day+42 post ASCT using both SPE/IFE and MALDI-TOF MS. The residual monoclonal protein was quantifiable in 2/3 (66.7%) of these patients by SPE and 3/3 (100%) using MALDI-TOF MS (residual monoclonal protein levels detectable by MALDI-TOF MS 0.23- 0.97 g/L).

At day+100 post ASCT, residual quantifiable monoclonal protein was detectable in 1/4 (25.0%) samples by SPE. Residual monoclonal protein was detectable in 2/4 (50.0%) patients using MALDI-TOF MS but in only 1/2 (50.0%) patients was the monoclonal protein detectable by MALDI-TOF MS present in a sufficient concentration to be quantitated (15 mg/L).

Confirmation of a CR according to the IMWG uniform response criteria requires the demonstration of <5% plasma cells in a bone marrow biopsy (Kumar et al., 2016). Bone marrow confirmation confirming CR was available for 1/2 (50.0%) of patients with no residual monoclonal protein detectable at the end of induction chemotherapy. The second patient had no bone marrow biopsy results available as part of the central laboratory analysis and could therefore only be classified as having achieved a VGPR. MRD results from the end of induction were not available for either patient. After 41.0 months follow-up, the patient who achieved a CR and MALDI-TOF MS negativity

remains alive and progression free; the patient who achieved a VGPR had a PFS of 35.1 months.

2/2 (100%) patients that were MALDI-TOF MS negative at day+100 post ASCT achieved a CR. MRD results were available for both patients and 1/2 (50.0%) were MRD positive. The patient who achieved a CR and MALDI-TOF MS negativity but was MRD positive had a PFS of 38.1 months. The patient who was in CR and in whom there was no residual disease detectable by MALDI-TOF MS or bone marrow MRD analysis by flow cytometry is alive and progression free after 78.0 months follow-up.

Samples from the time of disease progression were available for three patients. 3/3 (100%) of patients had intact immunoglobulin secreting disease detectable by SPE and MALDI-TOF MS at presentation. At disease progression 2/3 (66.7%) had intact immunoglobulin monoclonal protein detectable by SPE and 2/2 (100%) had monoclonal protein of the same isotype and in the case of MALDI-TOF MS with the same *m/z* identified at progression as that identified in the baseline sample. The third patient relapsed with a light chain escape pattern (no detectable monoclonal protein by SPE, serum free lambda 413.37 mg/L, serum FLC ratio 0.05). At the time of initial diagnosis of multiple myeloma this patient had a normal serum FLC ratio (serum free kappa 15.1 mg/L, serum free lambda 14.9 mg/L, sFLC ratio 1.015) and there were no detectable monoclonal lambda light chains in the urine using electrophoretic techniques but there were low-level monoclonal lambda FLC detectable by MALDI-TOF MS. At the time of relapse there was a 0.23 g/L IgG lambda monoclonal protein detectable by MALDI-TOF MS and monoclonal lambda FLC, which had the same *m/z*

as the IgG lambda monoclonal protein identified in the sample from presentation (Figure 5.4). These results are in keeping with their being a lambda FLC only secreting subclone at presentation that was only detectable by MALDI-TOF MS.

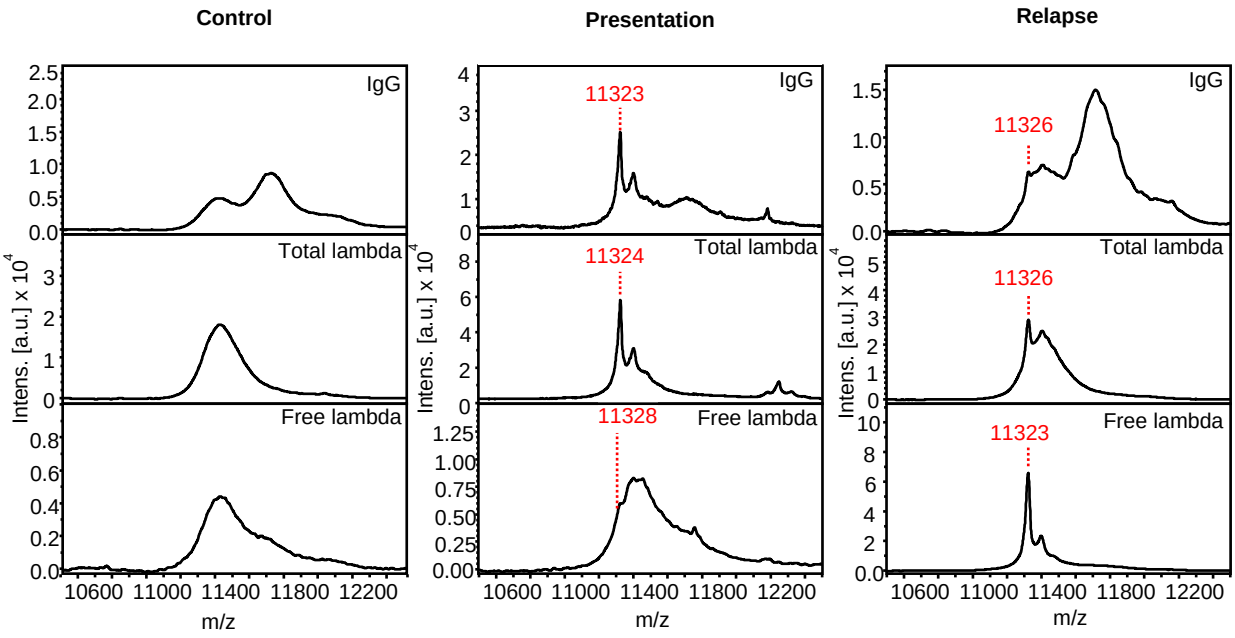


Figure 5. 4 MALDI-TOF MS detected low-level FLC production in patient with a normal sFLC ratio who relapsed with light chain escape
An IgG lambda monoclonal protein was detectable by MALDI-TOF MS and low-level monoclonal lambda FLC were detectable using FLC specific antisera (serum free lambda 14.9 mg/L, sFLC ratio 1.02) at presentation. At relapse, the patient had evidence of FLC escape (no detectable monoclonal protein by SPE/IFE, serum free lambda 413.4 mg/L, sFLC ratio 0.05). MALDI-TOF MS detected monoclonal lambda FLC with the same m/z as the low-level monoclonal lambda FLC detected at baseline and a 0.23 g/L IgG lambda monoclonal protein with the same m/z as the IgG lambda monoclonal protein identified at initial diagnosis. The monoclonal protein peaks are highlighted by the red dashed lines and mass labels in the mass spectra from presentation and relapse.

5.3.8 MALDI-TOF MS Can Be Used to Detect a VGPR in Patients with Oligo-secretory Multiple Myeloma

To assess the suitability of MALDI-TOF MS for assessing VGPR in patients with oligo-secretory multiple myeloma, samples from six patients with oligo-secretory multiple myeloma (monoclonal protein levels 2.55 g/L – 9.3 g/L) were diluted into pooled serum collected from healthy blood donors mixed with PBST (IgG 4.7 g/L, IgA 1 g/L and IgM 0.5 g/L, serum free kappa 4.99 mg/L and serum free lambda 7.35 mg/L). In addition,

two monoclonal proteins spiked into pooled serum from healthy blood donors at 1 g/L were assessed using dilutions into pooled serum from healthy blood donors. Monoclonal protein of the same isotype and with the same *m/z* as that identified in the baseline sample was detectable in all samples following 1:2 and 1:10 dilutions (Figure 5.5), indicating that MALDI-TOF MS could be used to assess a VGPR in all of these patients.

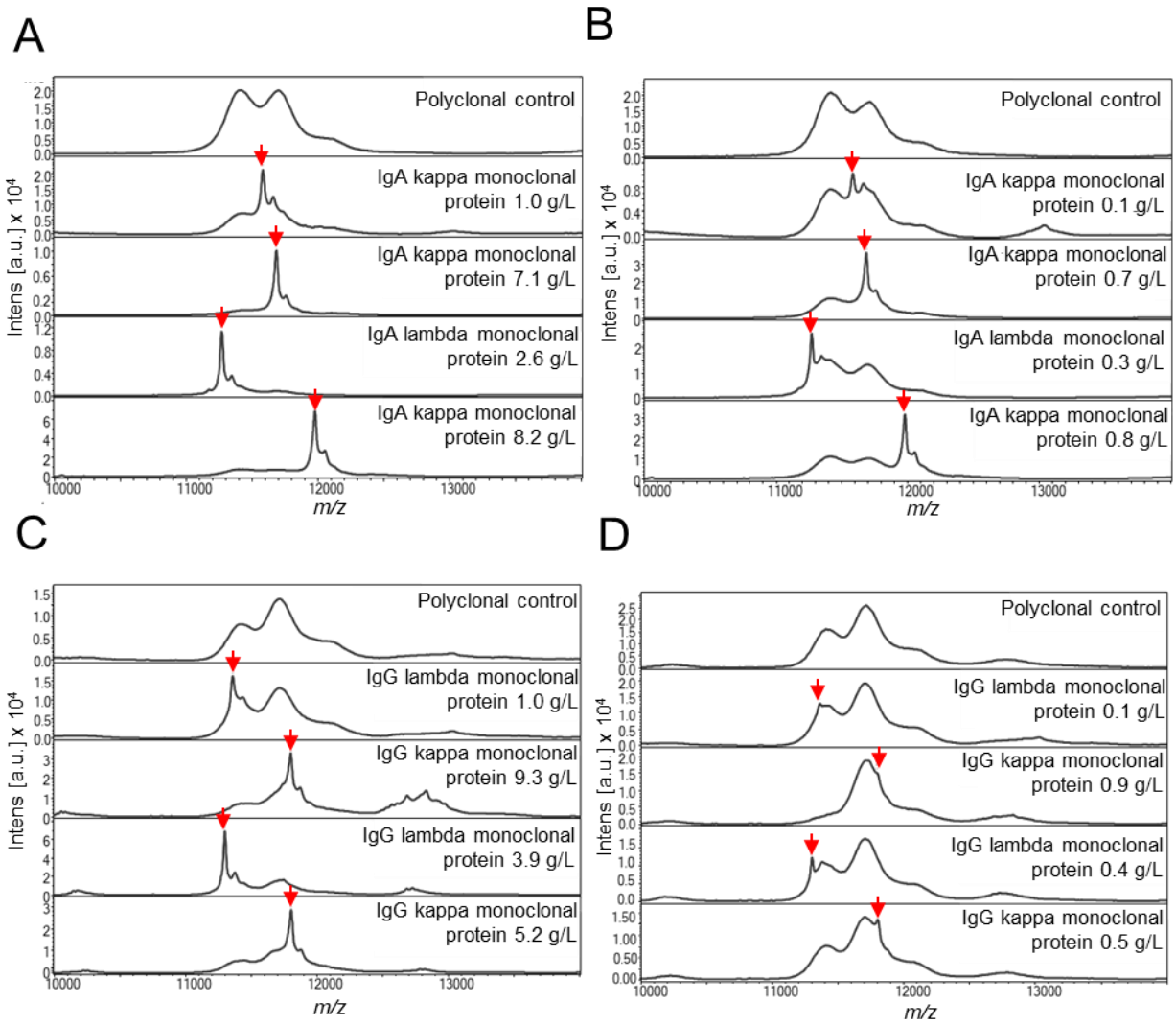


Figure 5. 5 MALDI-TOF MS can detect VGPR in patients presenting with monoclonal proteins with oligo-secretory multiple myeloma
MALDI-TOF MS identified monoclonal proteins of the same isotype as SPE/IFE in eight samples from patients with IgA and IgG monoclonal proteins <10 g/L (highlighted with the red arrows) in (A) and (B). Residual monoclonal protein with the same isotype and m/z was detectable in all samples following 1:10 dilution into healthy donor serum mixed with PBST (C) and (D).

5.4. Discussion

El Khoury *et al* reported a high rate of transient low-level monoclonal proteins, particularly IgM monoclonal proteins, in their analysis of samples from the PROMISE study (El-Khoury *et al*., 2022). The good agreement observed between the isotype of the monoclonal protein identified by MALDI-TOF MS and the light chain expression

pattern on the neoplastic plasma cells in patients with available histological results in supports that these low-level proteins are being produced by the neoplastic cells. In addition, the prevalence of IgM monoclonal proteins identified in this study was low (an IgM monoclonal protein was identified as a secondary monoclonal protein in 1/52 (1.9%) patients) as would be expected for patients with multiple myeloma in whom IgM monoclonal proteins are rare as these tend to be associated with low-grade lymphomas rather than PCD.

In this study, monoclonal protein was detectable using MALDI-TOF MS in the serum of 90.9% of presentation samples from patients with NSMM in the Myeloma XI trial and 60.0% of patients from UHB who presented with symptomatic PCD but had no detectable monoclonal protein by IFE. The inclusion of FLC specific antisera enhanced the sensitivity of MALDI-TOF MS for the detection of monoclonal protein at diagnosis, particularly in patients categorised as having NSMM based on electrophoretic results. 4/22 (18.2%) patients with NSMM treated in the Myeloma XI trial only had detectable monoclonal protein at presentation by FLC-MS and in 7/20 (35.0%) of the follow-up samples with detectable residual monoclonal protein by MALDI-TOF MS this was only detectable by FLC-MS. LC-MS also provided greater sensitivity for the detection of low-level intact immunoglobulin residual disease.

In keeping with the results of historical studies using immunohistochemical analysis and flow cytometry, this study provides further evidence that true NSMM due to absolute non-production of immunoglobulin by the neoplastic plasma cell clone is a very rare entity (Turesson and Grubb, 1978, Corso and Mangiacavalli, 2017). This

study has also highlighted that some patients with very low-level monoclonal protein production have intact immunoglobulin secreting disease.

The current criteria for measurable disease are based on the limit of detection of SPE and also take into account the difficulties in accurately being able quantitate low level monoclonal proteins as they may fail to resolve into a distinct band. In keeping with the results of preliminary laboratory studies, which have suggested that the QIP-MS/EXENT assay provides greater sensitivity than IFE for the detection (Ashby JA, 2018, Sakrikar *et al*., 2021), the work presented here demonstrates that the QIP-MS/EXENT assay provides greater sensivity for the detection of residual monoclonal protein in patients with oligo-secretory multiple myeloma and NSMM.

The serum FLC assays provide a valuable tool for monitoring both patients with FLC only multiple myeloma and patients with intact immunoglobulin monoclonal proteins where 95% of patients have evidence of FLC production and up to 10% may relapse with expansion of a predominantly FLC secreting subclone (Kühnemund *et al*., 2009, Brioli *et al*., 2014, Tacchetti *et al*., 2016, Tacchetti *et al*., 2022). In this study MALDI-TOF MS was able to both detect residual monoclonal FLC in patients in whom the FLC ratio had normalised and exclude residual disease in patients with abnormal serum FLC ratios in the context of suppressed uFLC, emphasising that direct measurement of the monoclonal FLC provides more accurate assessment of the presence of low-level residual disease. At present FLC-MS spectra are interpreted manually and in a qualitative fashion i.e. positive or negative, which limits throughput and means only binary result interpretation rather than quantitative assessments of

the percentage reduction in the monoclonal FLC can be determined using this assay. This means that the FLC-MS assay in its current form can detect a CR but cannot be used to define a PR or VGPR. The software that would perform automated quantitative analysis needs to be finalised so that further work can be undertaken to define what measurable disease using FLC-MS would be based on the sensitivity of the assay.

Although the preliminary sensitivity and linearity of quantitation data for the QIP-MS/EXENT assay look promising (Sakrikar *et al*., 2021), this assay needs to be studied in a greater variety of samples. Additional factors such the glycosylation status of the monoclonal light chain, the level of the polyclonal immunoglobulin background and the *m/z* of the monoclonal protein and its relative proximity to the apex may result in slight differences in assay sensitivity between samples. This work will be crucial to enable thresholds for measurable disease using MALDI-TOF MS to be defined.

Finally, comparisons between serological response assessments using MALDI-TOF MS and LC-MS and bone marrow MRD and functional imaging assessments need to be conducted to determine the utility of the assays for monitoring patients with non-measurable multiple myeloma and in which patients they may be most useful.

6:UTILITY OF FLC-MS FOR THE DETECTION OF MINIMAL RESIDUAL DISEASE IN THE PERIPHERAL BLOOD OF PATIENTS WITH SYSTEMIC AMYLOIDOSIS

6.1. Introduction

The introduction of serum FLC assays to the diagnostic armamentarium for the identification and monitoring of therapeutic responses in patients with AL amyloidosis represented a significant diagnostic advance. They provide a sensitive methodology for monitoring low-level monoclonal FLC in the blood and form the basis of the current criteria for haematological response (Comenzo *et al*., 2012). However, despite their sensitivity up to 20% of patients still have insufficient circulating monoclonal FLC levels for response assessment using serum FLC assessments (Dittrich *et al*., 2017, Nguyen *et al*., 2018b).

In addition, impaired renal function, which is commonly encountered in patients with AL amyloidosis, impacts the FLC level and ratio results and can present challenges in terms of result interpretation. The half-life of serum FLC is prolonged in patients with impaired renal function, which frequently results in higher circulating levels of polyclonal FLC (Hutchison *et al*., 2008b, Long *et al*., 2022). This can impact the sensitivity of FLC assays for the detection and monitoring of low-level monoclonal FLC

production (Palladini *et al*., 2016). It may also present challenges when applying dFLC criteria to assess for VGPR in patients with increasing degrees of severity of renal impairment.

Intact light chain MS assays, which are emerging as a novel and more sensitive methodology for monitoring monoclonal proteins in patients with PCD, have only been explored to a limited degree in patients with AL amyloidosis. The majority of studies have focussed on the presence or absence of N-linked light chain glycosylation, which has been reported in increased frequency in patients with AL amyloidosis (Milani *et al*., 2017b, Kumar *et al*., 2019, Mellors *et al*., 2021). To date, only one study by Dispenzieri *et al* has evaluated the potential prognostic utility of intact light chain MS assays for monitoring patients undergoing treatment for AL amyloidosis. In this study, Dispenzieri *et al* reported that residual monoclonal protein was detectable by MS in 12% of patients in CR and that MS positivity was associated with reduced time to progression (Dispenzieri *et al*., 2020a). Although the results of this study are encouraging, a combination of MALDI-TOF MS and LC-MS were used in this study and only 2/4 (50%) of MS positive patients had residual disease detectable by MALDI-TOF MS. The sensitivity of the MALDI-TOF MS assay (MASS-FIX) used in this study may not be optimal for monitoring for patients with AL amyloidosis as this assay does not include FLC specific reagents, which can enhance the sensitivity of MS assays for the detection of low-level residual monoclonal FLC.

The aims of this study were to:

- compare the sensitivity a MALDI-TOF MS assay using FLC specific reagents to FLC and electrophoretic assessments for the detection and monitoring of monoclonal proteins in patients undergoing treatment for systemic AL amyloidosis in the ALchemy study
- explore the prognostic utility of FLC-MS based response assessments in terms of OS and organ response in patients undergoing treatment for systemic AL amyloidosis in the ALchemy study

6.2 Methods

FLC-MS testing was performed in all available samples from patients treated in the ALchemy study in whom a baseline sample and a follow-up sample from either six or twelve months post treatment initiation were available for MS testing. The mass spectra were interpreted manually in a qualitative fashion i.e. positive or negative whilst blinded to the results of the electrophoretic and serum FLC assessments.

6.3 Results

6.3.1. Baseline Characteristics of the Patients from the ALchemy Study in whom FLC-MS Testing was Performed

The baseline characteristics of the patients included in this study are listed in Table 6.1.

Table 6. 1 Baseline characteristics of the patients from the ALchemy study included in this study of FLC-MS

Baseline Characteristic	All Patients (n=262)
Age, median (range)	67 (36-88)
missing, n (%)	136/262
Male, n (%)	157/262 (59.9)
Female, n (%)	105/262 (40.1)
Number of organs involved, n (range)	2 (1-4)
Renal Involvement, n (%)	194/262 (74.0)
Cardiac Involvement, n (%)	152/262 (58.0)
Bone marrow plasma cell percentage, median (range)	10 (0-90)
missing, n (%)	104/262 (39.7)
Kappa light chain AL amyloid	40/262 (15.3)
Lambda light chain AL amyloid	221/262 (84.4)
Biclonal AL amyloid	1/262 (0.4)
iFLC (mg/L), median (range)	189 (11.6-6407.0)
dFLC (mg/L), median (range)	160.7 (-14.8-6396.5)
dFLC <50mg/L, n (%)	55/262 (21.0)
BJP positive, n (%)	166/262 (63.4)
missing, n (%)	7/262 (2.7)
Serum creatinine (µmol/L), median (range)	95 (27-777)
eGFR (ml/1.73m2/minute), median (range)	68 (15-100)
missing, n (%)	5/262 (1.9)
NT-proBNP (pg/ml), median (range)	1394 (42-34082)

6.3.2. Sensitivity of FLC-MS for the Detection of Monoclonal Protein at Baseline in Comparison to Serum FLC Assays and Electrophoretic Techniques in Patients with AL Amyloidosis from the ALchemy Study

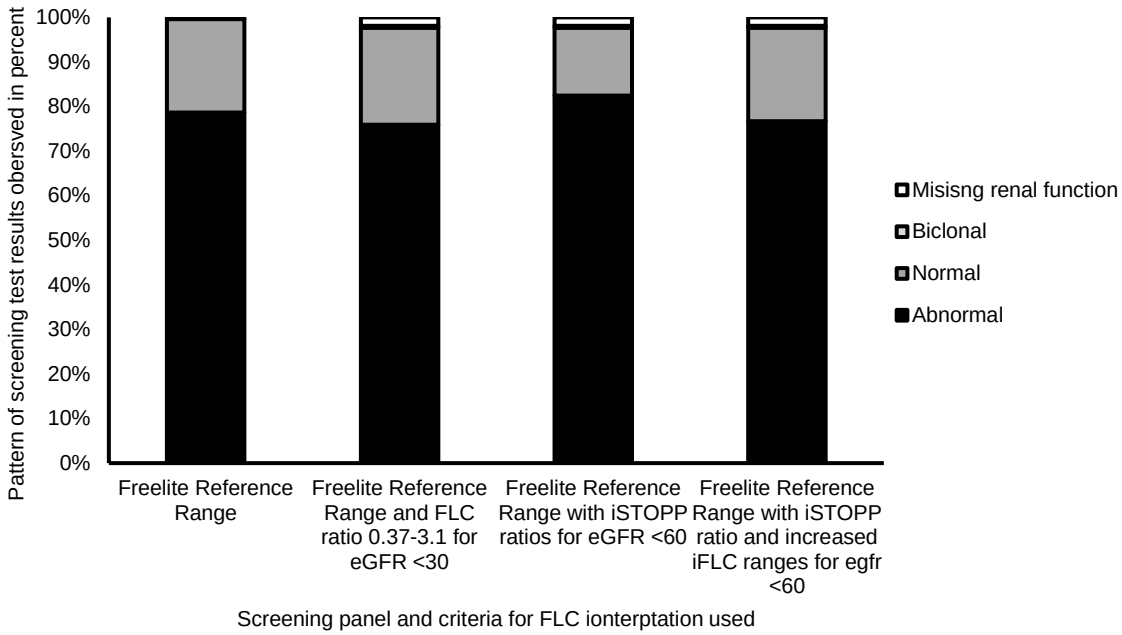
261/262 (99.6%) patients were classified as having a single involved light chain isotype using standard techniques and 1/262 (0.4%) patients had biclonal disease. Using the standard Freelite® reference range (Katzmann *et al*., 2002), the serum FLC ratio was abnormal 206/261 (78.9%) samples from patients with a single monoclonal protein. Renal function from baseline was available for 256/261 (98.1%) patients with a single monoclonal protein and when the renal reference of 0.37-3.1 proposed by Hutchinson *et al* was applied to patients with an eGFR <30ml/minute/1.73m^2 (Hutchison *et al*., 2008b), 199/256 (77.7%) had an abnormal serum FLC ratio. Using

the recently proposed 99th percentile ranges from the iSTOPMM study (Long *et al*., 2022) improved the sensitivity of the FLC ratio for the detection of low level monoclonal FLC – an abnormal serum FLC ratio was present in 216/256 (84.4%) patients when these FLC ratio reference ranges were applied to patients with an eGFR <60ml/minute/1.73m^2 (Figure 6.1A). However, 15/216 (6.9%) patients with an abnormal FLC ratio using the proposed iSTOPMM reference ranges (Long *et al*., 2022) did not have an elevated level of the iFLC.

55/262 (21.0%) of patients had a dFLC less than 50 mg/L at presentation and were classified as having low FLC burden AL amyloidosis. 29/55 (52.7%) patients with low FLC burden AL amyloidosis had a dFLC of 20-50 mg/L and were classified as having low dFLC AL amyloidosis. The remaining 26/55 (47.3%) patients with a dFLC <20 mg/L were classified as having non-measurable AL amyloidosis. BJP results were available for 54/55 (98.2%) patients with low FLC burden AL amyloidosis and 16/54 (29.6%) were BJP positive in their baseline urine sample. 44/55 (80.0%) of low FLC burden AL amyloidosis patients had monoclonal protein detectable by SPE and/or serum IFE and in 26/55 (47.3%) of these patients the monoclonal protein was ≥5 g/L.

Overall abnormal screening results were obtained in: 254/262 (96.9%) patients when FLC ratio (0.26-1.65 if eGFR ≥30ml/minute/1.73m^2 and 0.37-3.1 for patients with eGFR <30ml/minute/1.73m^2), IFE and BJP results were combined; and 256/262 (97.7%) when the iSTOPMM FLC ratio reference ranges were applied. Monoclonal FLC were detectable in 262/262 (100%) baseline samples using FLC-MS (Figure 6.1B).

A



B

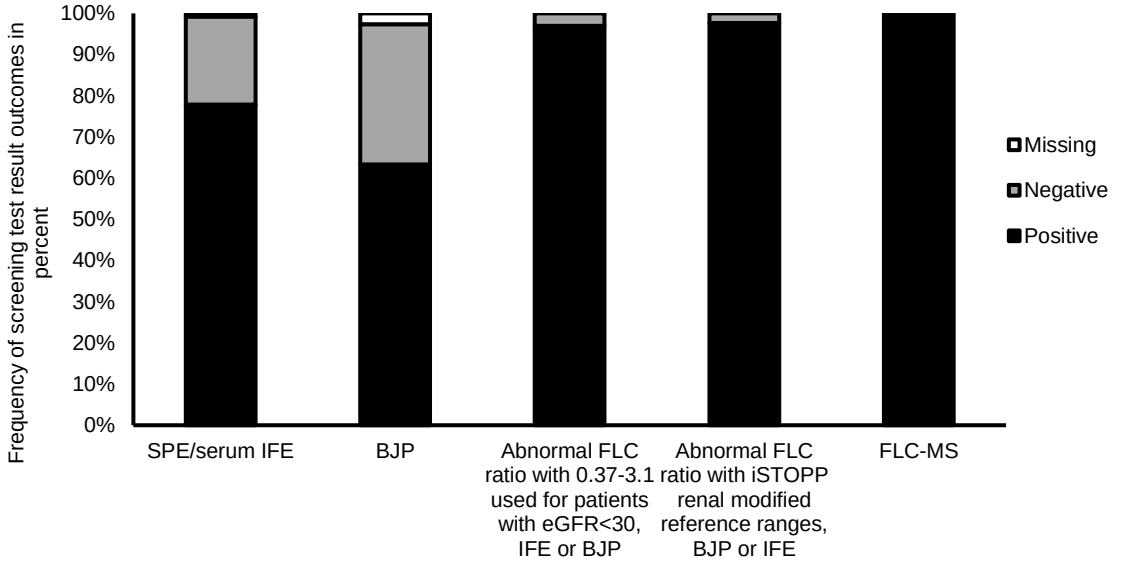


Figure 6. 1 Detection rates of monoclonal protein in baseline samples from patients in the ALchemy study using electrophoretic techniques, serum FLC assessments and FLC-MS

(A) Shows the detection rates for monoclonal FLC in the serum at diagnosis using the Freelite® assay with standard reference ranges and modified renal reference ranges. (B) shows the detection rates for monoclonal protein using electrophoretic techniques in the serum and urine and serum FLC assessments compared to FLC-MS in patients with systemic AL amyloidosis who were treated in the ALchemy study.

6.3.3 Residual disease detection by FLC-MS compared to Conventional Techniques at Six and Twelve Months Post Treatment Initiation in Patients Enrolled in the ALchemy Study

250/262 (95.4%) patients had paired haematological response assessments using conventional techniques and FLC-MS from six months post study enrolment. The results of the detection rates for the two methodologies are summarised in Figure 6.2. 78/250 (31.2%) patients achieved a haematological CR and 35/250 (14.0%) achieved a FLC-MS CR. Residual monoclonal FLC were detectable FLC-MS in 164/172 (95.3%) patients who were classified as being in less than a haematological CR using conventional techniques (Figure 6.2A).

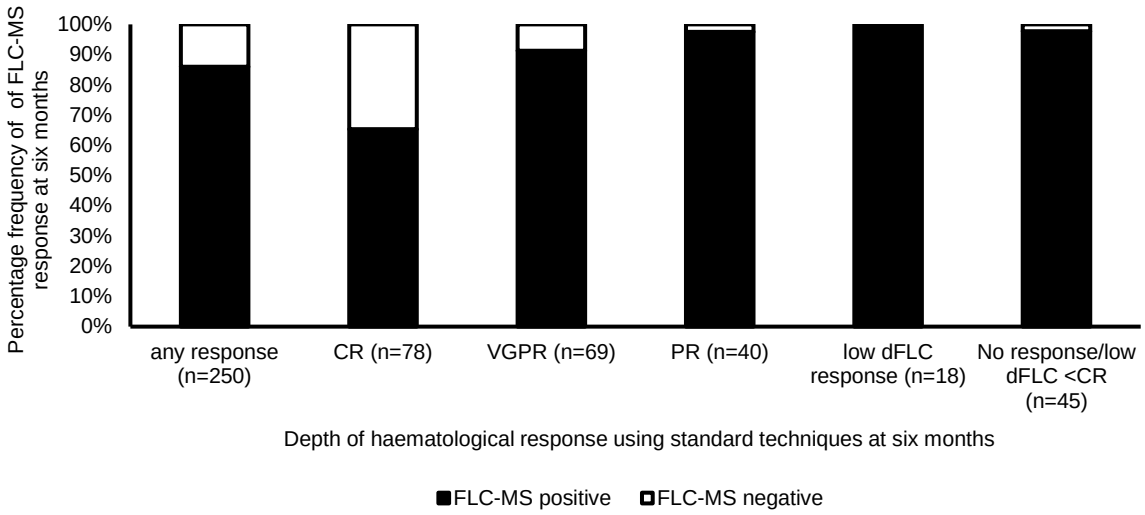
Amongst the eight patients in less than a haematological CR in whom there was no residual monoclonal FLC detectable by MS, 7/8 (87.5%) had a normal serum FLC ratio. 6/8 (75.0%) patients were classified as being in less than a CR due to residual positivity by SPE and/or serum IFE and 1/8 (12.5%) due to BJP positivity. 1/8 (12.5%) patients was classified as a PR due to dFLC of 46.3mg/L in the context of ongoing with renal impairment. 5/6 (83.3%) patients with persistent electrophoretic positivity in the serum had IgG monoclonal proteins and 2/5 (40.0%) converted to IFE negative by 12 months, suggesting the persistent IFE positivity at six months may have been due to the long half-life of the IgG monoclonal proteins.

254/262 (96.9%) patients had complete FLC-MS and standard haematological response assessments available from 12 months post study enrolment. Overall, 82/254 (32.3%) patients were in haematological CR and 48/254 (18.9%) were FLC-MS negative (Figure 6.2B). Residual monoclonal FLC was detectable by MS in

159/172 (92.4%) patients in less than a haematological CR and 47/82 (57.3%) of patients in CR.

3/13 (23.1%) patients who were FLC-MS negative but were classified as being in less than a haematological CR were only classified as being in less than CR due to incomplete electrophoretic results. 6/10 (60.0%) of the remaining MS negative patients in less than a haematological CR had abnormal serum FLC ratios and 3/6 (50.0%) of these patients had glycosylated light chains. 5/6 (83.3%) of the FLC-MS negative patients with a persistently abnormal FLC ratio had kappa light chain AL amyloidosis. 2/10 (20.0%) FLC-MS negative patients were BJP positive.

A



B

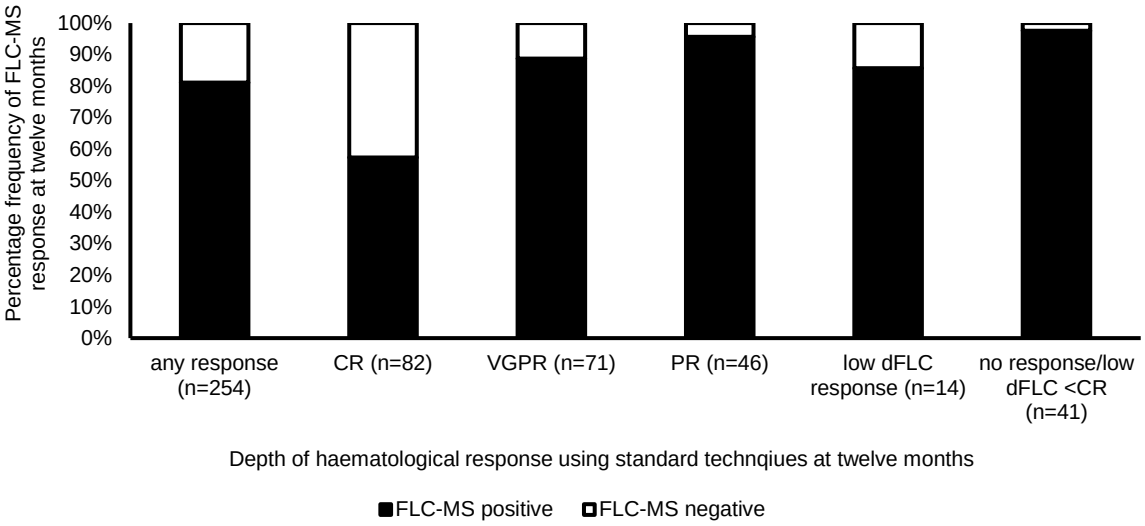


Figure 6. 2 Comparison between the detection rates of residual disease using standard haematological response criteria and FLC-MS in patients being treated for AL amyloidosis in the ALchemy study

In (A) the results from all patients with paired results from six months post study enrolment are shown and (B) shows the results from 12 months.

6.3.4. Prognostic Significance of FLC-MS Response

Negativity by FLC-MS at the six and 12 month time points was associated with improved OS (Figure 6.3A-B). When the analysis was restricted to patients in ≥VGPR, FLC-MS negativity at 12 months was also associated with a statistically significant improvement in OS (Figure 6.3D). However, at the six month time point there was only a non-significant trend towards improved OS for patients in ≥VGPR who were FLC-MS negative versus those with residual detectable monoclonal FLC by MS (Figure 6.3C).

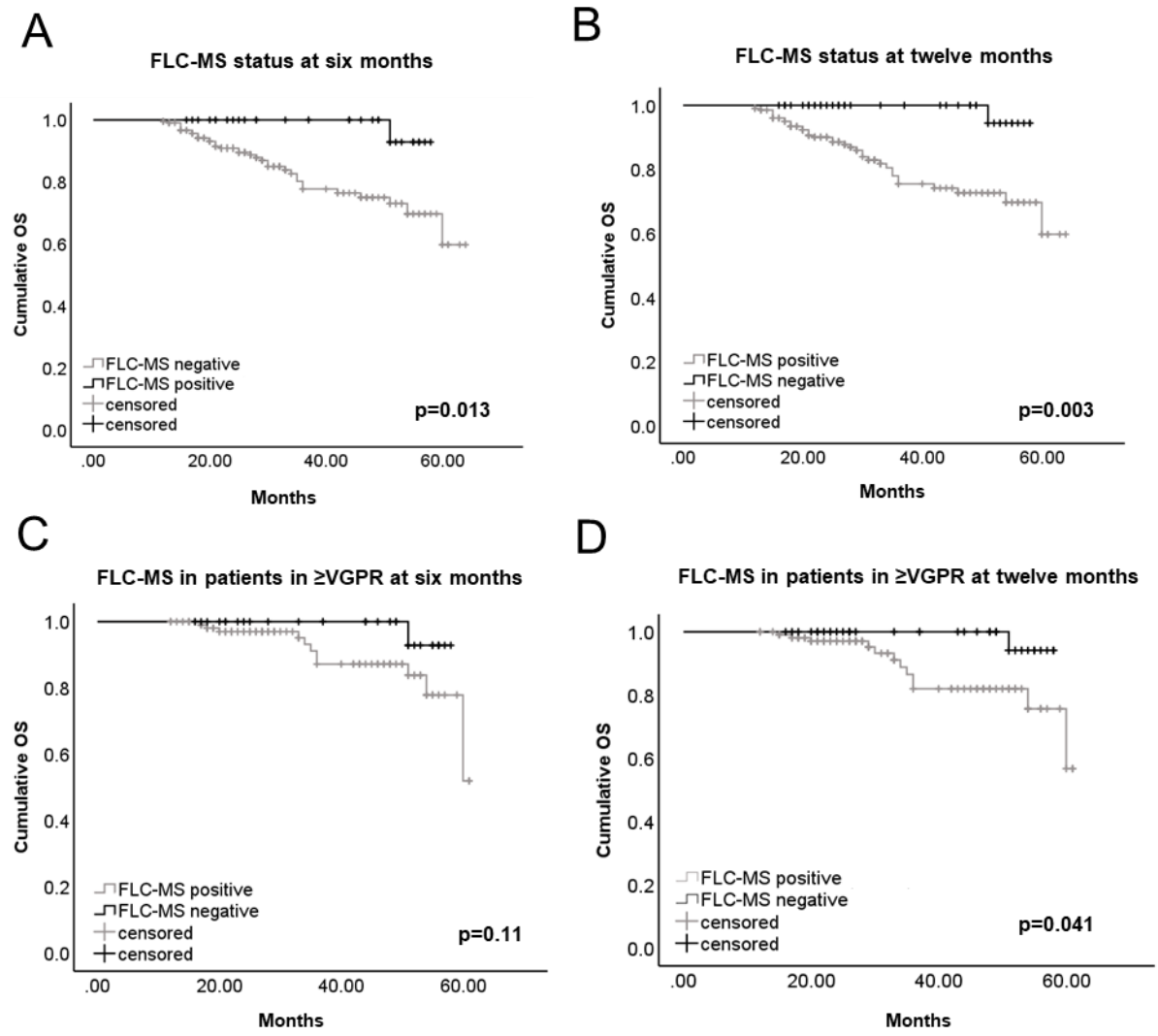


Figure 6. 3 OS according to FLC-MS response at six and twelve months post initiation of treatment for systemic AL amyloidosis.
(A) and (B) show the OS for FLC-MS positive versus negative patients at six and 12 months post study enrolment, respectively. (C) and (D) show the OS for MS positive versus negative patients who were in ≥VGPR at six and 12 months.

6.3.5 The Relationship between FLC-MS Response and Organ Response in Patients Undergoing Treatment for Systemic AL Amyloidosis in the ALchemy Study

166/194 (85.6%) patients with renal involvement had complete FLC-MS, renal function and proteinuria results from baseline and 12 months post study enrolment. There was a higher rate of renal response for patients who became FLC-MS negative using the renal response criteria from the consensus guidelines for the conduct and reporting of

clinical trials in systemic light-chain amyloidosis (Comenzo *et al*., 2012) (54.3% v 25.2%, p<0.01; Figure 6.4A). The statistically significant improvement in the renal response rate for FLC-MS negative patients also held true when the modified criteria for renal response proposed by Palladini *et al* were applied (Palladini *et al*., 2014) (Figure 6.4B).

169/194 (51.5%) patients with renal involvement had FLC-MS and proteinuria results available from baseline and 12 months post study enrolment and could be evaluated for renal progression based on the amount of proteinuria. A ≥30% increase in proteinuria that was ≥1g/24 hours occurred in 11/136 (8.1%) FLC-MS positive patients and 1/33 (3.0%) FLC-MS negative patients (p=0.52). Serum creatinine increases as a marker of renal progression were not evaluated as information regarding other factors which commonly effect serum creatinine levels, such as volume status and diuretic use, were not available.

131 patients had a baseline NT-proBNP ≥650 ng/L and had complete FLC-MS and NT-proBNP results from the 12 month timepoint. There was a non-significant trend towards an increased rate of cardiac response in patients who achieved FLC-MS negativity (57.7% v. 44.8%, p=0.34; Figure 6.4C). Cardiac troponin and ejection fraction results were not available, so the proportion of patients experiencing cardiac progression could not accurately be analysed.

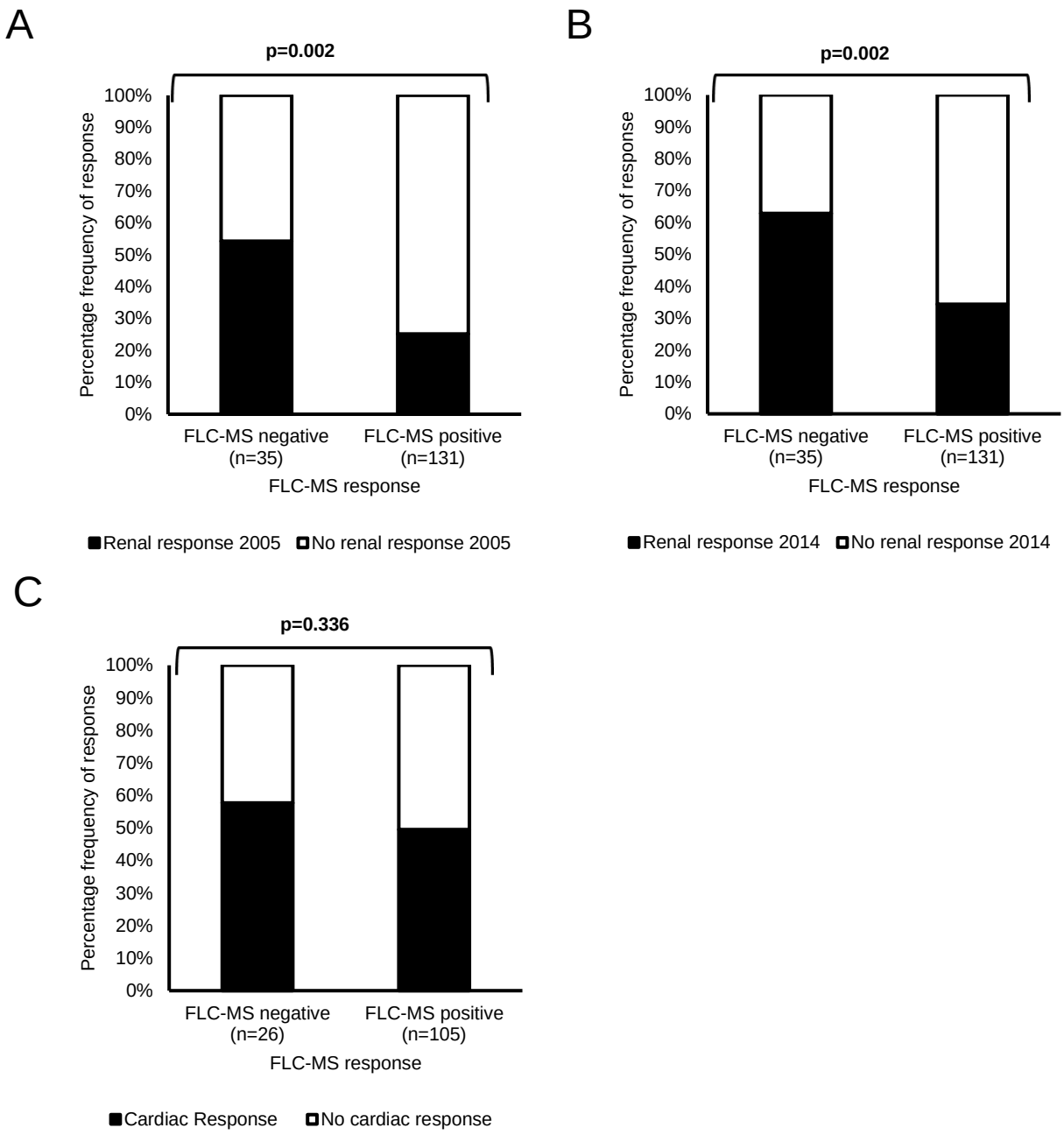


Figure 6. 4 The impact of FLC-MS status on organ response rates at 12 months post initiation of treatment for AL amyloidosis in Patients from the ALchemy study

The rates of renal response in FLC-MS negative and positive patients at 12 months post treatment initiation using the renal response criteria proposed by Comenzo et al (Comenzo et al., 2012) and the modified criteria proposed by Palladini et al (Palladini et al., 2014) are shown in (A) and (B) respectively. (C) shows the rates of cardiac responses observed in patients who were FLC-MS negative and FLC-MS positive at 12 months post treatment initiation.

6.4. Discussion

This study has shown that FLC-MS can offer a highly sensitive methodology for the detection and monitoring of monoclonal FLC in patients with AL amyloidosis with 100% sensitivity for the detection of monoclonal FLC in baseline samples. FLC-MS negativity was associated with improved OS and improved renal response rates.

The only intact light chain MS based assay that is currently being used in clinical practice for monitoring patients with PCD is the MASS-FIX assay which does not include FLC specific reagents and nor does the standard test set for the first commercialised MS assay under development. It will be important to understand how the relative sensitivity of the assays that only include total light chain reagents compares to assays using FLC specific reagents. This is particularly important as small numbers of samples from patients with FLC MGUS and AL amyloidosis who have been reported to be positive using current techniques have been negative by MASS-FIX (Wilhite *et al*., 2021, Mellors *et al*., 2021).

FLC-MS should also be compared against other sensitive techniques for monitoring patients who have achieved deep haematological responses, including electrophoretic techniques utilising FLC specific reagents (Singh and Bollag, 2020, Wilhite *et al*., 2021) and bone marrow MRD assays (Kastritis *et al*., 2021b, Li *et al*., 2021), to help determine the optimal monitoring strategy for patients undergoing treatment for AL amyloidosis.

Although this study provides further encouraging results to support the potential utility of MALDI-TOF MS assays in patients with AL amyloidosis, it does have some limitations. Firstly, assessments of cardiac response and progression were limited due to the lack of data on NYHA class response, ejection fraction and cardiac troponin results. Secondly, organ responses were only analysed at the 12 month time point but many studies have reported increasing organ response rates at later follow-up (Muchtar *et al*., 2021). This study may therefore have underestimated the impact of FLC-MS negativity on long-term organ responses. Thirdly, FLC-MS response assessments were graded categorically as positive or negative with no quantitative analysis. A better understanding of the amount of residual monoclonal FLC detectable may help further define depths of MS response and help elucidate different prognostic categories based on the depth of response.

Lastly, the addition of daratumumab to VCD has been shown to significantly improve haematological CR rates in the ANDROMEDA trial (Kastritis *et al*., 2021c), however no patients included in this study were treated with daratumumab-VCD as they were all treated prior to this regimen achieving NICE approval (NICE, 2024). The increased sensitivity afforded by MS may be particularly valuable in this context and may further separate out the depth of response amongst patients who have achieved a haematological CR using the standard techniques, which was over 50% of patients treated with Daratumumab VCD in the ANDROMEDA study (Kastritis *et al*., 2021c). The rates of FLC-MS CR and its prognostic utility should therefore be explored in future studies in patients treated with Daratumumab-VCD and the results should be compared to response assessments using the electrophoretic techniques in

combination with serum FLC assessments and bone-marrow based MRD assessments.

7:THE PREVALENCE AND CLINICAL SIGNIFICANCE OF LIGHT CHAIN N-LINKED GLYCOSYLATION IN PATIENTS WITH MULTIPLE MYELOMA AND AL AMYLOIDOSIS

7.1 Introduction

The presence of light chain N-linked glycosylation in the mass spectra of some patients with PCD has emerged as an area of interest, as it has been observed at increased frequency in patients with monoclonal proteins, particularly patients with AL amyloidosis. Amongst the patients with AL amyloidosis the increased prevalence of light chain N-linked glycosylation has been more marked in patients with kappa light chain AL amyloidosis where rates of up to 33% light chain N-linked glycosylation have been observed (Kumar *et al*., 2019, Mellors *et al*., 2021, Nevone *et al*., 2022, Dasari *et al*., 2021). However, it is less clear whether the incidence of light chain N-linked glycosylation is increased in patients with lambda light chain AL amyloidosis compared to patients with MGUS and multiple myeloma. Kumar *et al* reported light chain N-linked glycosylation in 10.2% of patients with lambda light chain amyloidosis compared to 4.9% of patients with lambda light chain myeloma (Kumar *et al*., 2019) but other studies have reported light chain N-linked glycosylation in patients with lambda light chain AL amyloidosis that are very similar to those seen in MGUS and multiple myeloma (Mellors *et al*., 2021, Nevone *et al*., 2022).

A recent study by Dispenzieri *et al* also reported that the presence of light chain N-linked glycosylation in patients MGUS was associated with an increased risk of progression to symptomatic PCD (Dispenzieri *et al*., 2020b), however no studies have evaluated the outcomes of patients with and without light chain N-linked glycosylation who are undergoing treatment for PCD.

The aims of this study were therefore to:

- Evaluate the prevalence of light chain N-linked glycosylation in patients with newly diagnosed multiple myeloma treated in the KCRD arm of the myeloma XI trial and evaluate whether its presence was associated with high risk features at presentation
- To evaluate whether patients with light chain N-linked glycosylation undergoing treatment for myeloma have inferior responses to treatment and/or shorter PFS in comparison to patients without light chain N-linked glycosylation
- To evaluate the prevalence of light chain N-linked glycosylation in patients with AL amyloidosis and to compare the prevalence to that seen in patients treated in the Myeloma XI study
- To evaluate whether the presence of light chain N-linked glycosylation in patients undergoing treatment for systemic AL amyloidosis in the ALchemy study was associated with any difference in haematological or organ response rates and OS.

7.2 Methods

The spectra obtained from the presentation samples from the 293 patents from the KCRD arm of the Myeloma XI trial described in chapter four and the 262 patients from the ALchemy study tested as part of chapter six were manually inspected for the presence of light chain N-linked glycosylation based on the presence of polytypic peaks with an increased *m/z* with peak differences consistent with N-linked glycans. For the samples from the Myeloma XI trial the spectra from the QIP-MS/EXENT and FLC-MS testing were evaluated and from the ALchemy study the spectra from FLC-MS testing only were inspected.

The baseline characteristics and survival data for the patients from the Myeloma XI trial were supplied by the CRUK Clinical Trials Units in Leeds and the baseline characteristics, treatment response and OS data for the patients from the ALchemy study were supplied by the National Amyloidosis Centre in London. Statistical analyses were performed as described in Chapter 2.

7.3 Results

7.3.1 Prevalence and Clinical Significance of Light Chain N-linked Glycosylation in Patients with Newly Diagnosed Multiple Myeloma who Were Treated with KCRD in the Myeloma XI Trial

Light chain N-linked glycosylation were observed in 26/293 (8.9%) patients. 1/293 (0.3%) had an equivocal result for a small secondary monoclonal protein with light chain N-linked glycosylation of a different isotype to main monoclonal protein identified by IFE and QIP-MS/EXENT. The presence of N-linked glycosylation of the light chain component of the monoclonal protein was confirmed in three samples using incubation

of the captured light chain on the magnetic microparticles with PNGase F prior to analysis by FLC-MS (Figure 7.1).

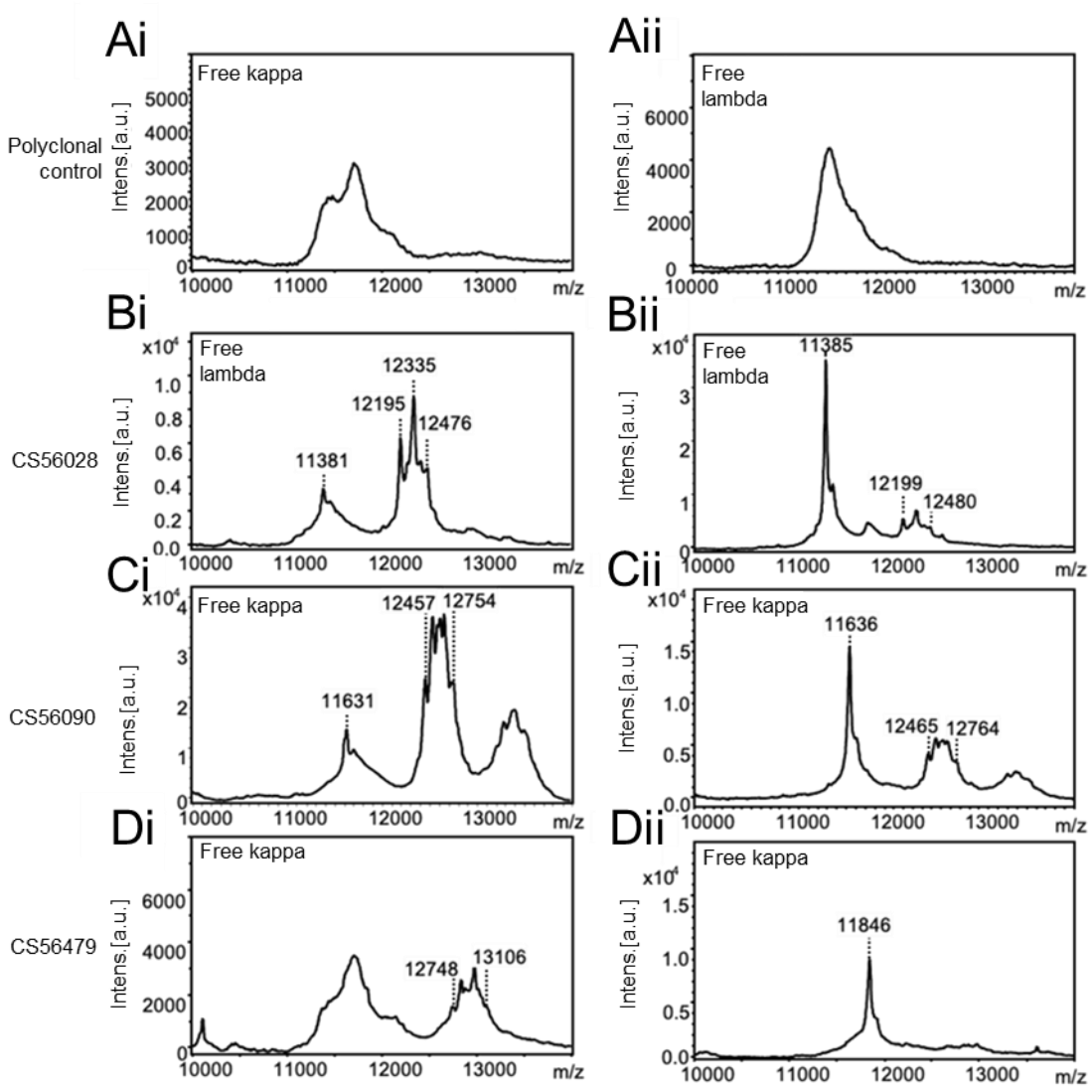


Figure 7. 1 Light chain N-linked glycosylation was identified based on the presence of polytypic peaks with an increased *m/z* and can be confirmed by performing on-bead de-glycosylation with PNGase F

(Bi-Di) show spectra with polytypic peaks suggestive of N-linked light chain glycosylation. (Bii-Dii) shows the m/z of the non-glycosylated form of the monoclonal FLC following on-bread de-glycosylation using PNGase F. Control mass spectra obtained from polyclonal serum are shown in (Ai) and (Aii). The CS codes refer to the unique clinical sample code assigned to the sample when the anonymised aliquot was booked in at the Binding Site Ltd.

The clinical and biochemical characteristics of the patients presenting with and without light chain N-linked glycosylation are summarised in Table 7.1. There was no significant difference in the biochemical disease burden, degree of plasma cell infiltration, haemoglobin level or platelet count between patients with and without N-linked light chain glycosylation. There was also no difference in serum beta-2-microglobulin or serum albumin levels.

The median creatinine level at presentation was lower in patients with light chain N-linked glycosylation. However, there was no significant difference in the level of the iFLC at presentation compared to patients without evidence of light chain glycosylation (480.5 mg/L v. 434.9 mg/L, p=0.93). In contrast, BJP levels were higher in patients without light chain N-linked glycosylation (0.08 g/L v. 0.02 g/L, p=0.02).

Table 7. 1 Baseline characteristics of the patients with and without evidence of N-linked light chain glycosylation on their monoclonal protein at presentation

	MP without Light Chain Glycosylation (n=266)	MP with Light Chain Glycosylation (n=26)	Statistical Significance
Age, years (range)	60.6 (33.7-80.6)	58.2 (37.6-69.8)	0.42
Monoclonal protein level, g/L (range)	33.3 (0.27-101.7)	32.1 (3.1-86.0)	0.812
iFLC level, mg/L (range)	434.9 (5.9-40347)	480.5 (0.37-10631)	0.932
BJP, g/L (range)	0.08 (0-122.12)	0.02 (0-3.28)	0.018
Median Haemoglobin, g/L (range)	112 (52-159)	112.5 (70-143)	0.946
Median serum creatinine (range)	94 (42-315)	79 (52-123)	0.011
Median platelet count, x 10^9/L (range)	254 (37-838)	243 (123-486)	0.73
Median percentage bone marrow plasma cell infiltration (range)	40.0 (0-100)	40 (5.0-89.0)	0.964
Median albumin level, g/L (range)	40.0 (18.7-52.6)	40.2 (24-50)	0.951
Beta-2-microglobulin, mg/L (range)	3.4 (1.1-30.1)	3.2 (1.7-7.8)	0.51
Patients with Lactate Dehydrogenase >220 IU/ml (%)	134/224 (59.8)	11/19 (57.9)	0.468
Patients with bone disease (%)	199/266 (74.8)	21/26 (80.8)	0.664

7.3.2 Treatment Responses and Survival Outcomes of Patients in the KCRD Treated Arm of the Myeloma XI Trial Presenting With and Without Light Chain N-linked Glycosylation

Results for the central response analysis were available for 287/292 (98.3%) patients from the end of induction and 256/292 (87.7%) patients from day+100 post ASCT. MRD results from the end of induction and day+100 post ASCT were available in 131/292 (44.9%) and 166/292 (56.8%) of the patients respectively. In this cohort of myeloma patients outcomes were similar between patients with and without light chain N-linked glycosylation (Figure 7.2). There was also no difference in the depth of response at the end of induction treatment with KCRD or at day+100 post high dose melphalan conditioned ASCT between patients with and without light chain N-linked glycosylation (57.7% ≥nCR v. 55.2% ,nCR, p=0.97 and 69.6 v. 67.4%, p=0.98 at the end of induction and day+100 post ASCT respectively). In addition, in patients with available MRD results the proportion of patients achieving MRD negativity did not significantly differ (70.0% v. 62.8%, p= 0.91 and 71.4% v. 73.7%, p=0.50 at the end of induction and day+100 post ASCT respectively).

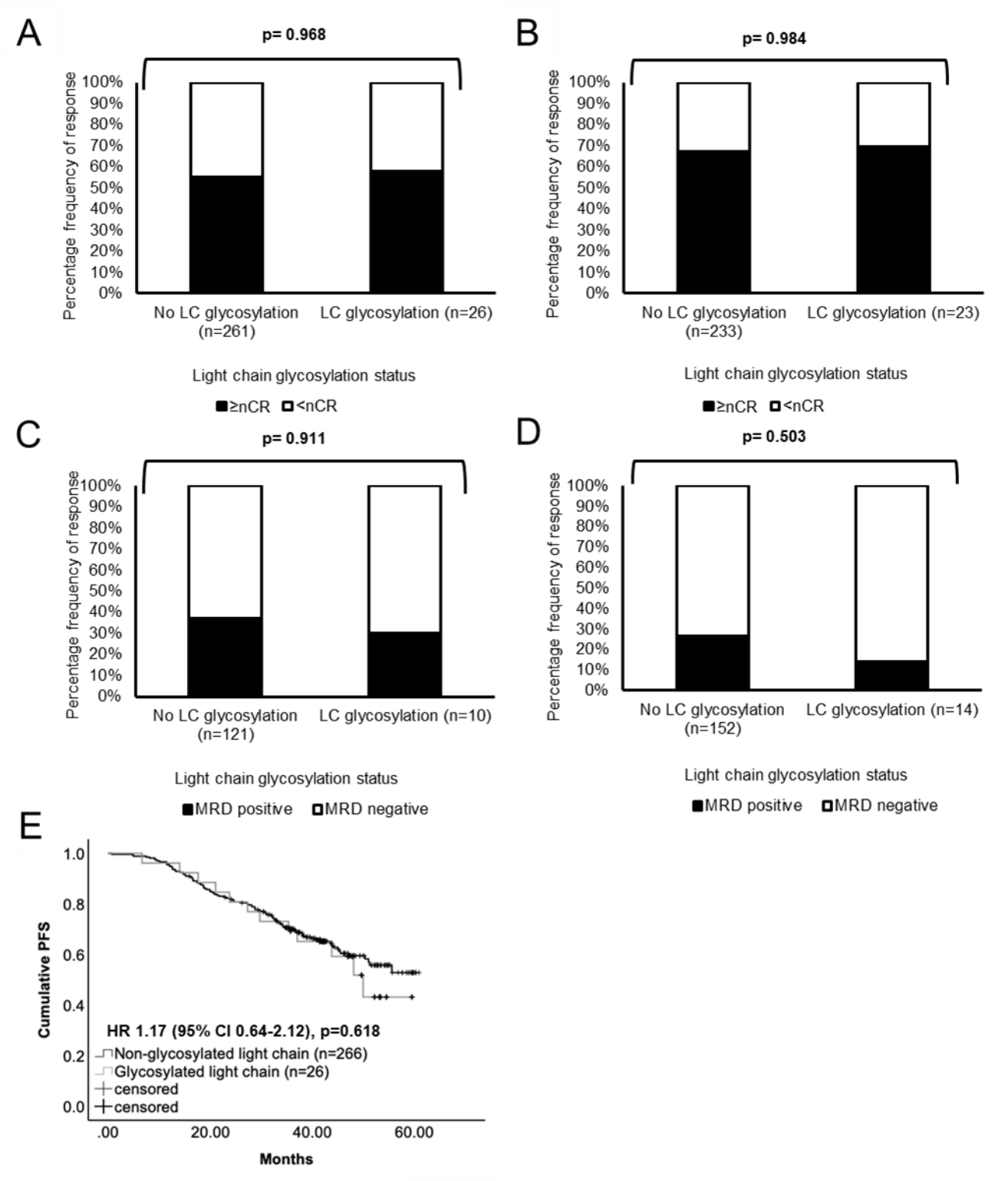


Figure 7. 2 Depth of response and PFS according to light chain glycosylation status in patients treated in the KCRD arm of the Myeloma XI trial.

Proportion of patients achieving ≥nCR at the end of induction (A) and (B) day +100 post ASCT. (C-D) show the proportion of patients achieving MRD negativity at the end of induction and day+100 post ASCT respectively. (D-E) show the PFS of patients with and without light chain glycosylation. The significance of the differences between the observed frequencies have been compared using Chi squared tests with Yates's correction applied.

In keeping with the lack of difference in depth of response, light chain N-linked glycosylation was not associated with reduced PFS (median PFS 50.0 months v. NR, p=0.62 for patients with and without light chain N-linked glycosylation, respectively; Figure 7.2E). It is unclear whether the contrasting outcome results reported here compared to the study by Dispenzieri *et al* are because light chain glycosylation is more of a high-risk feature in non-myeloma PCD as over half of the progression events in patients with light chain N-linked glycosylation in the study by Dispenzieri *et al* were to non-myeloma symptomatic PCD (Dispenzieri *et al*., 2020b). The limited duration of follow-up in this study may have also limited the ability to detect any difference and it is important to note that in the study by Dispenzieri *et al* the difference in progression did start emerging until at least 10 years after initial diagnosis of MGUS.

7.3.3 Prevalence and Clinical Characteristics of Patients with AL Amyloidosis and Light Chain N-linked Glycosylation

36/262 (13.7%) patients had mass spectra consistent with N-linked glycosylation of the monoclonal FLC. The presenting characteristics of the patients with and without light chain N-linked glycosylation are summarised in Table 7.2. FLC glycosylation was more prevalent in patient with kappa light AL amyloidosis; 17/40 (42.5%) patients with kappa light chain had FLC chain glycosylation versus 19/221 (8.6%) patients with lambda producing AL amyloidosis. Neither monoclonal FLC in the patient with biclonal disease had evidence of N-linked glycosylation. There were no statistically significant differences in organ tropism between patients with and without FLC glycosylation, although there was a non-significant trend towards increase liver involvement in patients with light chain N-linked glycosylation.

In contrast to the observation of improved renal function at presentation observed in the patients from the Myeloma XI trial who had light chain glycosylation, there was no statistically significant difference in the renal function at presentation between patients with and without light chain N-linked glycosylation in this cohort of patients with AL amyloidosis.

Table 7. 2 Baseline characersitics of patients with and without N-linked glycosylation of the monoclonal FLC

	Light Chain N-linked Glycosylation (n=36)	No Light Chain N-linked Glycosylation (n=226)	*p value*
Bone marrow plasma cell infiltration (%)	10 (2.5-60)	10 (0-90)	0.347
Kappa light chain amyloidosis, n (%)	17/36 (47.2)	23/226 (10.2)	<0.001
Lambda light chain amyloidosis, n (%)	19/36 (52.8)	202/226 (89.4)	
Biclonal, n (%)	0/36 (0.0)	1/226 (0.4)	
iFLC (mg/L), median (range)	242.4 (17.7-1330.0)	183.7 (11.6-6407.9)	0.395
Serum creatinine (umol/L), median (range)	97 (42-541)	95 (27-777)	0.81
eGFR (ml/minute/1.73m^2), median (range)	65 (15-100)	68 (15-100)	0.653
NT-proBNP (pg/ml), median (range)	1128 (110-9159)	1394 (42-34082)	0.841
Renal involvement, n (%)	23/36 (63.9)	171/226 (75.7)	0.196
Cardiac involvement, n (%)	23/36 (63.9)	129/226 (57.1)	0.557
Liver involvement, n (%)	5/36 (13.9)	10/226 (4.4)	0.06
GI involvement, n (%)	1/36 (2.8)	10/226 (4.4)	0.992
Soft tissue involvement, n (%)	4/36 (11.1)	39/226 (17.3)	0.495
Autonomic nervous system involvement, n (%)	1/36 (2.8)	15/226 (6.6)	0.601

7.3.4 Comparison of the Prevalence of Light Chain N-linked Glycosylation in Patients with AL Amyloidosis in the ALchemy Study and Patients with Multiple Myeloma from the KCRD Arm of the Myeloma XI Trial

The comparison between the rates of light chain N-linked glycosylation observed in in patients with AL amyloidosis from the ALchemy study and patients with multiple myeloma from the KCRD arm of the Myeloma XI trial are shown in Figure 7.3. Overall there was a statistically non-significant trend towards an increased rate of light chain N-linked glycosylation in patients with AL amyloidosis compared to the rate observed in myeloma patients (13.7% v. 8.9%, p=0.10). When analysing the difference according to the light chain isotype, light chain N-linked glycosylation was significantly more frequent in patients with kappa AL amyloidosis compared to patients with kappa light chain myeloma (42.5% v. 10.8%, p<0.01) but there was no difference in the rate of light chain glycosylation detected in patients with lambda light chain AL amyloidosis compared to patients with lambda light chain myeloma (8.6% v. 5.7%, p=0.48).

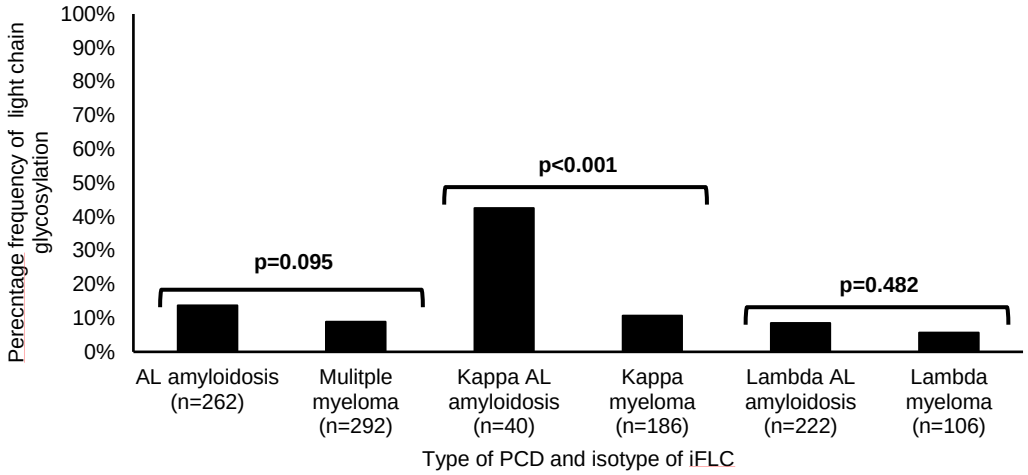


Figure 7. 3 The rates of light chain glycosylation according to type of PCD and isotype of the iFLC

The percentage frequency of light chain N-linked glycosylation observed in patients from the ALchemy study and the KCRD treated arm of the Myeloma XI trial are shown in this bar chart. The monoclonal protein isotype has been categorised as per the MS results. The significance of the differences between the observed frequencies have been compared using Chi squared tests with Yates's correction applied.

7.3.5 Treatment Responses and OS Outcomes of Patients with AL Amyloidosis in the ALchemy Study Presenting with and Without N-linked Light Chain Glycosylation

There was no difference in the rate of haematological CR at 12 months between patients with and without FLC N-linked glycosylation (Figure 7.4A). However, there was a non-significant trend towards an increased rate of MS CR in patients with FLC glycosylation (Figure 7.4B).

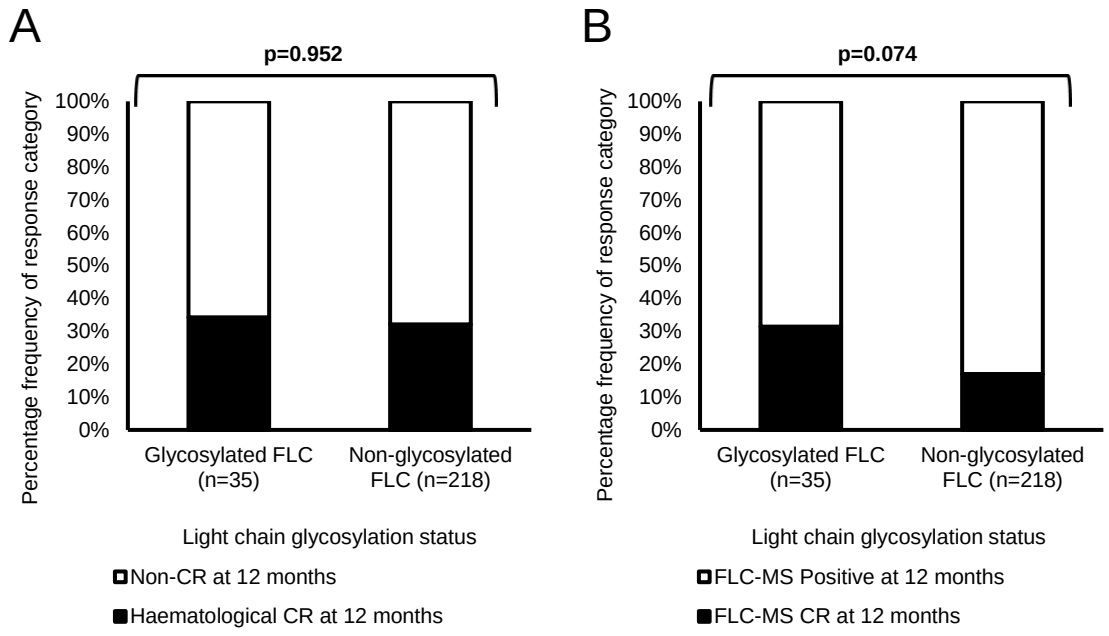


Figure 7. 4 Impact of light chain glycosylation status on the depth of response and OS.

The rates of haematological CR and FLC-MS CR at 12 months post study enrolment for patients with and without FLC glycosylation are shown in (A) and (B). The significance of the differences between the observed frequencies have been compared using Chi squared tests with Yates's correction applied.

A potential explanation for the trend toward a higher MS CR rate in patients with FLC chain glycosylation is that the sensitivity of FLC-MS may be lower due to the broader nature of the peaks in patients with FLC glycosylation.

After a median follow-up of 31 months, there was no significant difference in OS between patients with and without FLC glycosylation (Figure 7.5A). FLC glycosylation status had no impact NT-proBNP response rates (Figure 7.5B) but there was a non-significant trend towards an increased likelihood of a renal response in patients with FLC glycosylation (63.2% v. 37.2%, p=0.054; Figure 7.5C).

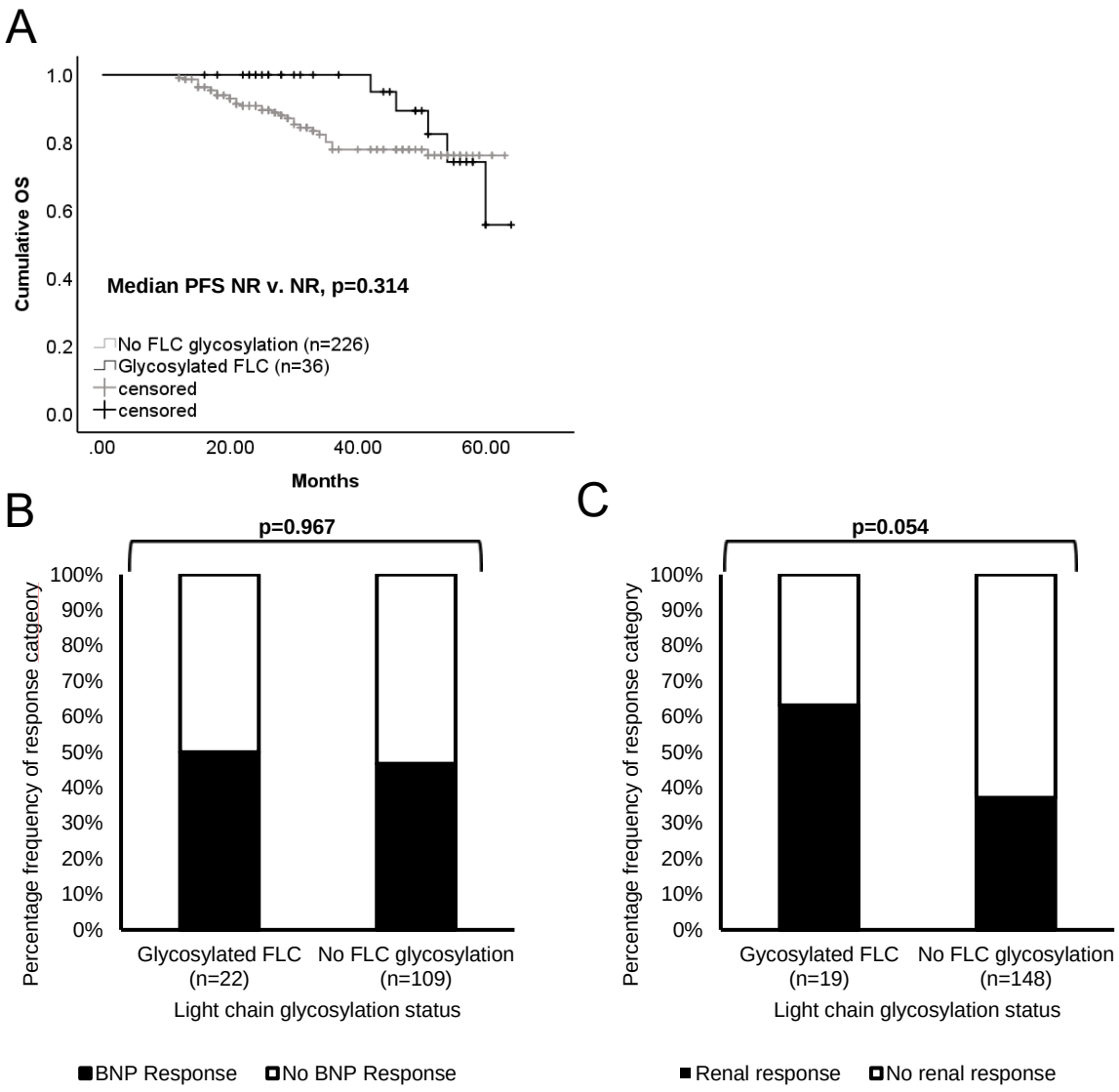


Figure 7. 5 Impact of FLC glycosylation status on OS and Organ Response Rates at 12 months in Patients form the Alchemy study
(A) shows the OS in patients with and without light chain N-linked glycosylation. (B) shows the NT-proBNP response in patients with and without light chain N-linked glycosylation and (C)shows the renal response rates at 12 months in patients with and without light chain N-linked glycosylation. The significance of the differences between the observed frequencies have been compared using Chi squared tests with Yates's correction applied.

7.4 Discussion

This study is the first to evaluate the relationship between light chain N-linked glycosylation and presenting features and clinical outcomes in patients with newly diagnosed multiple myeloma and AL amyloidosis. In contrast to the high-risk phenotype reported in MGUS patients by Dispenzieri *et al* (Dispenzieri *et al*., 2020a),

the presence of light chain N-linked glycosylation in patients undergoing treatment for symptomatic multiple myeloma was not associated high risk disease features in this study. Due to the number of patients with missing genetic results, this prognostic variable was not evaluated in this work but should be included in other studies evaluating the clinical significance of light chain N-linked glycosylation in patients with PCD.

In patients with multiple myeloma the presence of light chain N-linked glycosylation was associated lower BJP levels and lower creatinine levels but there was no significance difference in the level of the iFLC in the serum at presentation between patients with and without light chain N-linked glycosylation. It is possible that the complex branched structures of the N-linked glycans have altered light absorption properties in solution compared to non-glycosylated light chains, which could lead to over-estimation of the light chain levels by the Freelite® assay. Alternatively, light chains with N-linked glycosylation could be less prone to cast formation and therefore the level at which they cause renal injury may be higher.

The presence of light chain N-linked glycosylation in transplant-eligible patients undergoing treatment for newly diagnosed multiple myeloma was not associated with reduced rates of nCR or MRD negativity. In addition, there was no difference in PFS between patients with and without light chain N-linked glycosylation. The impact of light chain N-linked glycosylation on OS will be evaluated once longer follow-up data is available.

Similar to the results reported in other cross-sectional studies of samples from patients with AL amyloidosis (Kumar et al., 2019, Mellors et al., 2021, Dasari et al., 2021, Nevone et al., 2022), an increased rate of light chain N-linked glycosylation was observed in patients with kappa AL amyloidosis. However, there was no significant increase in the rate of light chain N-linked glycosylation inpatients with lambda AL amyloidosis compared to patients with myeloma in which the involved light chain isotype was lambda. There has been interest in whether light chain glycosylation may provide a useful marker to aid in the earlier identification of patients with AL amyloidosis (Dispenzieri et al., 2020b, Kumar et al., 2019). The results of the work presented in this chapter suggest that whilst the presence of light chain N-linked glycosylation may be helpful in identifying patients with potential kappa light chain amyloidosis, which less commonly the iFLC in patients with AL amyloidosis (Mellors et al., 2021), it will not help in identifying patients with lambda light AL amyloidosis. Alternative assays, such as Amylite (Jiang et al., 2023), may be more beneficial to assist in the earlier identification of patients with lambda light chain AL amyloidosis. It is important to note that over half of patients with kappa AL amyloidosis do not have light chain N-linked glycosylation, so the absence of light chain glycosylation cannot be used to exclude kappa AL amyloidosis, and kappa light chain N-linked glycosylation is also observed in patients with non-AL amyloid PCD. Future studies should evaluate whether the presence of light chain N-linked glycosylation can be combined with other simple screening tests such as NT-proBNP levels and ACR screening to assist in the earlier diagnosis of patients with kappa light chain AL amyloidosis.

In contrast to the finding observed in the myeloma patients of lower creatinine levels in patients with light chain N-linked glycosylation, no significant difference between the

creatinine levels observed in patients with AL amyloidosis with and without light chain N-linked glycosylation was observed. A comparison between the BJP levels in patients with and without light chain N-linked glycosylation could not be undertaken as part of this work as only qualitative results stating whether BJP were present or not and the isotype of the BJP were available.

FLC N-linked glycosylation was not associated with adverse outcomes in terms of haematological response, OS or organ responses in patients with AL amyloidosis. In contrast, there was a non-significant trend towards increased renal responses in patients with light chain N-linked glycosylation. The association between light chain N-linked glycosylation and organ responses should be evaluated in larger cohorts of patients with AL amyloidosis to establish whether the lack of statistical significance observed in this study was due to the relatively small number of patients with light chain glycosylation included in this study.

In conclusion, whilst the results of this work are encouraging that the presence of light chain glycosylation is not associated with adverse outcomes in patients presenting with symptomatic PCD, further work needs to be done to verify these findings. This is particularly relevant as the follow-up duration in this study was relatively short and the evaluation of light chain glycosylation in patients with multiple myeloma only included younger, fitter transplant patients. Larger scale studies should be easier to undertake now that the development of the fully automated workflow, including analysis of the MS spectra, has been completed for the QIP-MS/EXENT assay and optimisation work

has improved the batch size for FLC-MS testing from 40 to 72 samples per run, although this assay does still require manual interpretation of all spectra at this stage.

8:GENERAL DISCUSSION AND CONCLUSIONS

8.1. Summary of Results

This research project has investigated the utility of intact light chain MS-based assays for the detection and monitoring of monoclonal proteins in patients with symptomatic PCD.

The main results from the work included in this thesis are:

1. The sensitivity of intact light chain MALDI-TOF MS assays for the detection of residual monoclonal protein in patients who have undergone systemic treatment for multiple myeloma is enhanced by the inclusion of FLC-specific antisera

2. Oligoclonal peaks are highly prevalent in mass spectra even prior to ASCT and therefore careful consideration needs to be given to the timepoint of the sample used to identify the isotype and *m/z* of the monoclonal protein to be tacked by MALDI-TOF MS

3. On-bead de-glycosylation improves the sensitivity of FLC-MS for the detection of residual monoclonal FLC in patients who presented with glycosylated monoclonal FLC

4. FLC-MS negativity in IFE negative patients who have undergone treatment for newly diagnosed symptomatic multiple myeloma is associated with improved PFS but normalisation of the serum FLC ratio is not

5. MALDI-TOF MS provided greater sensitivity compared to electrophoretic techniques and serum FLC assays for the detection of monoclonal proteins in patients undergoing systemic treatment for non-measurable myeloma

6. FLC-MS provided greater sensitivity for the detection of monoclonal FLC in patients with AL amyloidosis at presentation and during follow-up following treatment

7. FLC-MS negativity was associated with improved OS and an improved renal response rate in patients undergoing treatment for AL amyloidosis in the ALchemy study

8. Light chain N-linked glycosylation was not associated with inferior PFS in patients undergoing systemic treatment for multiple myeloma or OS in patients undergoing treatment for AL amyloidosis.

8.2. Discussion and Conclusions

The main objective of this study was to determine whether MS-based assays provide greater sensitivity for the identification and monitoring of monoclonal proteins in patients with PCD compared to the current electrophoretic techniques and serum FLC assays. This was explored in three patients cohorts: transplant-eligible patients undergoing systemic treatment for newly diagnosed symptomatic multiple myeloma in the Myeloma XI trial; patients with non-measurable myeloma; and patients undergoing

systemic treatment for AL amyloidosis in the ALchemy study. In all three disease settings, residual monoclonal protein was detectable in a greater proportion of patients compared to the standard serological techniques.

In patients undergoing systemic treatment for multiple myeloma, MALDI-TOF MS negativity was associated with improved PFS. However, patients who achieved MS negativity but had early re-emergence of their monoclonal protein had similar outcomes to MS-positive patients. This finding is in keeping with the results reported in studies looking at bone marrow MRD status, where sustained negativity is associated the best outcomes (Munshi *et al*., 2017, Gu *et al*., 2018, Martinez-Lopez *et al*., 2020, Avet-Loiseau *et al*., 2021, Mohan *et al*., 2022, Paiva *et al*., 2023, Fonseca *et al*., 2023). At all three time points FLC-MS positivity identified patients with no detectable monoclonal protein by IFE who were at increased risk of progression.

At all time points the most common reason for discrepancies between QIP-MS/EXENT and IFE was MS positivity in IFE negative patients, which is as expected given the higher sensitivity of the MALDI-TOF MS based assay. However, amongst the IFE negative patients the commonest reason for discrepancies between FLC-MS assessments and serum FLC ratio results differed over time. At the end of induction chemotherapy and at day+100 post ASCT the most common reason for discordant results was FLC-MS positivity in samples with a normal serum FLC ratio, however at six months post maintenance randomisation over half of the discrepant results were due to an abnormal serum FLC ratio in patients who were FLC-MS negative. In addition, the distribution of the causes for the abnormal serum FLC ratio differed

between the follow-up time points. For example, at the end of induction chemotherapy and day+100 post ASCT 68.4% and 33.3% of cases in which the serum FLC ratio was abnormal but FLC-MS was negative were due to suppression of the uFLC but at the six months post maintenance time point none of the patients with an abnormal FLC ratio who were FLC-MS negative had an abnormal FLC ratio due to suppression of the uFLC. This is likely reflective of a greater degree of treatment related immune suppression when patients were receiving quadruplet induction and in the early phase post ASCT. In contrast at the later timepoint post maintenance randomisation a greater degree of oligoclonal immune reconstitution would have been occurring.

When analysing the trends across serial samples from individual patients, the concordance patterns varied over time in many patients. For example, within the 80 patients who were IFE negative at the end of induction and day+100 post ASCT who had paired FLC-MS and FLC ratio results at both time points, 31/80 (38.8%) had a different concordance pattern at the two time points. The changing patterns of concordance were due to a variety of reasons, including achieving FLC-MS negativity post ASCT as a deepening of the FLC-MS response post ASCT but developing an abnormal FLC ratio whilst others remained positive by FLC-MS but the FLC ratio normalised post ASCT. These discrepant results likely reflect the differential sensitivity between the assays and the varying degrees of immune reconstitution occurring over time between different patients.

It is challenging to know what the most appropriate reference range is to use for serum FLC ratio interpretation as many amended reference ranges have been proposed

since the launch of the Freelite® assay (Hutchison *et al*., 2008b, Molina-Andújar *et al*., 2020, Long *et al*., 2022, Morales-García *et al*., 2023). If the reference ranges for FLC ratio result interpretation recently proposed by the iSTOPMM group (Long *et al*., 2022, Einarsson Long *et al*., 2023) were applied to the IFE negative patients who were FLC-MS negative but had an abnormal FLC ratio, 66.7% and 63.6% of the FLC ratio results from day+100 post ASCT and six months post maintenance randomisation would be reclassified as normal and would therefore have been concordant with the negative FLC-MS result.

The interpretation of serum FLC results is more challenging in patients with renal impairment and several amended renal references ranges have been proposed since the introduction of serum FLC assays (Hutchison *et al*., 2008b, Molina-Andújar *et al*., 2020, Long *et al*., 2022). The ability of the MS assays to specifically track the monoclonal FLC rather than relying on an indirect measure in the form of a skewed FLC ratio is likely to be particularly valuable in patients with renal impairment where uncertainty about the optimal normal range for the FLC ratio at varying degrees of renal impairment exists. The study evaluating the potential utility of MS in patients treated with KCRD in the Myeloma XI trial only included a very small number of patients with an eGFR <30 ml/minute/1.73m^2 and therefore the improved clinical performance of FLC-MS may have been underestimated in this work. Analysis of the utility of FLC-MS in patients with severe renal impairment was not conducted as part of this thesis but future studies including patients with PCD and severe renal impairment are planned. These studies will include evaluating samples from patients treated in the EuLITE study (Hutchison *et al*., 2019) and samples from patients with monoclonal gammopathy of renal significance.

The commonest reason for discrepant results between combined testing with QIP-MS/EXENT and FLC-MS and bone marrow-based MRD assessments at the end of end of induction, day+100 post ASCT and six months post maintenance randomisation was MS positivity in patients who were MRD negative patients. Whilst some of the cases of discrepant results between MS and bone-marrow based MRD assessments will have been due to the long half-life of IgG, this does not explain all of the discrepant results because discordant results were observed in patients with non-IgG monoclonal proteins as well as those with IgG monoclonal proteins. In addition, in over half of the cases where discrepant results were observed between bone marrow-based MRD assessment and MS, residual disease was detectable by FLC-MS in addition to by QIP-MS/EXENT and the half-life of FLC is only a few hours. The relatively low sensitivity of the flow cytometry based MRD assay used in the Myeloma XI trial (de Tute *et al*., 2016) may have also contributed to some of the discrepant cases where the MS assays were positive for residual disease but the bone marrow testing was negative.

A direct comparison between the sensitivity of the bone marrow MRD based assays and the peripheral blood MS-based assays is complicated by the fact that the two types of assay use different units of measurement – the bone marrow-based assays are measured as the number of residual neoplastic cells per total number of nucleated cells analysed whereas the MS-based assays are measuring protein and the units of measurement are mg/L or g/L. In addition the bone marrow-based MRD assays sample a single site whereas the peripheral blood MS-based assays provide a global

assessment and may therefore detect residual disease that does not involve the posterior superior iliac crest sampling site, which is most commonly used to obtain bone marrow aspirate samples for MRD testing.

In an effort to overcome the issue of false negative MRD results due to single site biopsies for the bone marrow MRD assays, studies have explored the utility of functional imaging assessments to supplement bone marrow based MRD assessments. In these studies, although concordance between functional imaging assessments with PET-CT and/or DW-MRI and bone-marrow based MRD assessment has been low, the two modalities for MRD assessment do appear to be complementary as the best outcomes have been reported for patients achieving double negativity (Moreau *et al*., 2017, Rasche *et al*., 2019, Alonso *et al*., 2019, Zamagni *et al*., 2023). The overall reported rates of MRD positivity by imaging in patients who are negative for MRD in the bone marrow is relatively low at 6-21% patients (Rasche *et al*., 2019, Alonso *et al*., 2019, Böckle *et al*., 2022), but its frequency is increased in patients treated for relapsed disease (Rasche *et al*., 2019) and discrepancies are more common with patients with extra-medullary disease or para-skeletal plasmacytomas at presentation (Paiva *et al*., 2020). The concordance between MRD assessments in the bone marrow by flow cytometry and/or NGS, by functional imaging assessments and by MS should be explored in future studies. This would help determine how many discrepant results between MS and bone marrow based MRD assessments are due to residual disease not involving the posterior superior iliac spine sampling site.

It would be important that future studies comparing the MS assays to bone marrow-based MRD assays studies include MRD assays with a minimum sensitivity of $1x10^{-5}$ to ensure that this meets the current IMWG standard for an MRD assay in multiple myeloma (Kumar *et al*., 2016). However, ideally assays with a sensitivity of $1x10^{-6}$ would be used as negativity at this threshold has been shown to be associated the best outcomes (Martinez-Lopez et al., 2020, Costa et al., 2021b, Martinez-Lopez et al., 2024). Serial time points should be evaluated in these future studies as when using the bone marrow-based MRD assays persistence of negativity has been shown to be associated with best patient outcomes (Munshi *et al*., 2017, Gu *et al*., 2018, Martinez-Lopez *et al*., 2020, Avet-Loiseau *et al*., 2021, Mohan *et al*., 2022, Fonseca *et al*., 2023, Paiva *et al*., 2023).

There are currently several ongoing trials exploring the utility of MRD to guide consolidation and maintenance strategies (Royle *et al*., 2022, Derman *et al*., 2022), however none of these trials have yet incorporated MS-based response assessments. If they were it would be important to consider the impact of the long half-life of intact immunoglobulin monoclonal proteins as the work presented in this thesis highlight that there are still a significant number of patients, particularly those with IgG monoclonal proteins, that convert to MALDI-TOF MS negativity in the first year of maintenance treatment. If earlier time points for MS analysis were to be included in future studies in this area, the utility of FLC-MS alone versus QIP-MS/EXENT could be explored.

It is important to consider that although overall patients with multiple myeloma who have detectable residual disease have inferior outcomes compared to patients who

achieve MRD negativity, there are a subset of patients who maintain prolonged responses with detectable residual disease (Vu *et al*., 2015, Paquin *et al*., 2020). This is likely due to several patient and disease-related factors which may impact the significance of residual disease between patients.

Firstly, the nature of the residual disease will differ between patients and it has been shown that patients who are MRD positive with an MGUS-like phenotype have good outcomes (Gozzetti *et al*., 2022). In contrast, patients with high risk disease who have persistent MRD positivity do particularly badly (Paiva *et al*., 2020). Even when they achieve MRD negativity several studies have found that they remain at higher risk of relapse (Kunacheewa et al., 2020, Costa et al., 2021b), although this conclusion has not been universally reported (Perrot *et al*., 2018, Paiva *et al*., 2020).

Secondly, the functionality of the immune system and its ability to control low-levels of residual disease differs between patients and it has been shown that long-term survivors have an immunological profile in keeping with a less suppressed immune system (Bryant *et al*., 2013). Several studies have also reported that recovery of polyclonal immunoglobulins is associated with improved PFS (Harutyunyan *et al*., 2016, González-Calle *et al*., 2017, Michallet *et al*., 2018). The QIP-MS/EXENT assay quantifies the polyclonal background as levels of heavy light chain pairs and therefore the potential utility of monitoring these levels during maintenance should be explored to see whether they can help differentiate different prognostic groups within the patients with persistent MS-positivity.

Due to the limited number of samples available in patients with non-measurable myeloma, the prognostic implications of MALDI-TOF MS-based assessments in this patient cohort were not assessed. However, the results look encouraging and this technology may provide a useful adjunct to guide the timing of more invasive response assessments and aid in the early identification of patients with minimally secretory PCD. Previously published sensitivity results for the QIP-MS/EXENT assay have reported a lower limit of quantitation of 15 mg/L (Sakrikar et al., 2021), however the dilution series performed in this project demonstrate that assay sensitivity is likely to vary based on a variety of factors including the proximity of the monoclonal protein to the apex of the polyclonal background and the glycosylation status of the monoclonal protein. In addition to these factors, the level of the background polyclonal immunoglobulins is also known to impact the sensitivity of the MS-based intact light chain assays (Murray, 2022). These variables need to be evaluated across a broader range of samples, so that a greater understanding of the minimum levels of monoclonal protein detectable by MS that would be sufficient to permit MS monitoring versus those patients in whom MS positivity would simply provide a marker to guide timing of other response assessments such as bone marrow biopsies.

In addition to obtaining more detailed information on sensitivity, further prospective studies comparing MS-based assays to imaging and serial bone marrow assessments in patients with non-measurable myeloma need to be conducted. Clinical studies evaluating the utility of MALDI-TOF MS assays for relapse detection also need to be performed as this was not evaluated in this work as there were no data available on surveillance imaging and/or bone marrow biopsies performed post maintenance randomisation for patients with non-measurable myeloma. These studies would likely

require a collaborative international effort as these patients are currently excluded from most clinical trials, so stored serial samples may be a rare resource.

The work presented in chapter six is only the second study to look at the prognostic utility of the intact light chain assays for response assessment in AL amyloidosis and is considerably larger than the previous study, which only included in 33 patients (Dispenzieri *et al*., 2020a). Due to the increased sensitivity provided by using FLC specific antisera, the work presented in this thesis was able to demonstrate that MALDI-TOF MS alone was able to provide greater sensitivity than serum FLC and electrophoretic assessments in patients with AL amyloidosis and that FLC-MS negativity at 12 months translated in improved OS for all patients and those who had achieved at least an amyloid VGPR. This is important as in the small study by Dispenzieri *et al* the results of MALDI-TOF MS and LC-MS were combined for the survival analysis (Dispenzieri *et al*., 2020a) and the current LC-MS techniques are unsuitable for large-scale routine clinical use due to long-processing times and the expertise required to process the samples and analyse the data (Murray *et al*., 2021). In addition, the upfront instrumentation cost for LC-MS analysers is much more substantial.

The work in chapter six is also the first work to evaluate the significance of MS-based response assessments on organ response and demonstrated that MS negativity was associated with improved renal responses. It is well recognised that organ responses take significantly longer to achieve than haematological responses in patients with AL amyloidosis (Muchtar *et al*., 2018), therefore larger numbers of patients and longer

follow-up are required to determine whether FLC-MS status is predictive of the likelihood of other organ responses and this should be evaluated in future studies using FLC-MS.

Although FLC-MS positivity was associated with poorer outcomes overall in the samples analysed from patients from the ALchemy study, the rate of residual MS positivity was high and some patients with residual positivity still achieved organ responses and durable survival. The FLC-MS spectra generated in this work were only analysed qualitatively i.e. FLC-MS positive or FLC-MS negative, which means that patients could only be defined as having achieved a FLC-MS CR or positive for residual disease and therefore there was no differentiation between patients in a PR, VGPR and those with progressive disease by FLC-MS. Quantitative analysis and defining a VGPR, PR and progressive disease by FLC-MS may help further separate out FLC-MS positive patients into different prognostic groups based on the level of residual detectable monoclonal FLC. Similar to the further work that needs to be undertaken in patients with non-measurable myeloma, further work including quantitative analysis of FLC-MS results in patients with AL amyloidosis needs to be undertaken to establish criteria for measurable disease using FLC-MS in patients with AL amyloidosis.

The work presented in chapter seven is the first study to explore the prognostic implications of light chain N-linked glycosylation in patients undergoing treatment for symptomatic PCD. In contrast to the increased risk of progression from MGUS to symptomatic PCD described by Dispenzieri *et al* (Dispenzieri *et al*., 2020b), there was

no evidence of light chain glycosylation being associated with reduced PFS in the patients with new diagnosed symptomatic multiple myeloma or AL amyloidosis analysed in this work. In addition, no statistically significant association with other high risk biomarkers in patients with multiple myeloma was identified in this work. The reason for these contrasting results is unclear. If the higher rate of progression observed in the study by Dispenzieri *et al* (Dispenzieri *et al*., 2020b) was due to increased inflammation in biologically older individuals with a greater degree of immune dysregulation, this would not have been reflected in this current work as only younger fitter transplant-eligible patients from the Myeloma XI study were included in this work.

A limitation of the work include in this thesis is that none of the patients with multiple myeloma and AL amyloidosis received treatment that included an anti-CD38 monoclonal antibody. The addition of anti-CD38 monoclonal antibodies has been shown to provide deeper responses (Voorhees *et al*., 2020, Menon *et al*., 2021, Kastritis *et al*., 2021c) and therefore a higher rate of MS-negativity would be expected. Samples from patients receiving regimens including anti-CD38 therapies should therefore be included in future MS-based studies of patients with PCD.

Although a significant number of the myeloma patients included in this work received maintenance with lenalidomide, none of the AL amyloid patients included in this work received maintenance, which is now standard of care for patients with newly diagnosed systemic AL amyloidosis following the approval of Daratumumab-VCD by NICE in 2024 (NICE, 2024). The kinetics of MS-based responses in patients being

treated for AL amyloidosis during induction, post ASCT where applicable and during maintenance, should be studied to help identify the optimal timepoint/s for performing FLC-MS testing.

The potential utility of MS to enable earlier relapse detection was not explored in any of the clinical studies included in this thesis as only a small number of samples from disease progression were analysed as part of the laboratory work undertaken for this thesis. This should therefore be evaluated in future studies, particularly in patients with PCD associated with lower level monoclonal protein production including non-secretory myeloma, oligo-secretory myeloma, AL amyloidosis and patients with plasmacytomas.

The increased cost of the MS-based assays in comparison to the standard electrophoretic-based techniques and serum FLC assays is likely to have a significant impact on how widely the intact light chain MS-based assays may be adopted in routine clinical practice. Whilst the work presented in this thesis has shown that the MALDI-TOF MS based assays provide greater sensitivity in patients with measurable myeloma, non-measurable myeloma and AL amyloidosis, the cost of differential of utilising the MS assays compared to how these cohorts are currently monitored differs significantly. Owing to the need to perform more bone marrow and imaging studies in patients with non-measurable myeloma, routine monitoring of these patients is more expensive and less convenient for patients than simple blood tests so this would make a logical initial cohort for the implementation of MS-based assays. The implementation of MS testing in this patient cohort would require the establishment of criteria for

measurable disease for the MS-based assays. Similarly, the initial implementation of MS in AL amyloidosis may be targeted at the patients who are the most challenging to monitor, including patients who present with a dFLC <20mg/L at presentation and no measurable monoclonal protein by SPE and patients with significant renal impairment in whom serum FLC results can be particularly difficult to interpret.

Even if the MS assays were to be more widely adopted in patients with measurable disease, given the higher costs of the MS-based assays (Wilhite *et al*., 2021, Giles *et al*., 2022) it would be more cost effective to perform initial routine monitoring with these assays and only reflex to MS-based testing once a deep serological response has been observed. Based on the observations in the KCRD treated patients from the Myeloma XI trial where there were a small number of patients who had low-level residual monoclonal proteins reported by SPE and were classified as having achieved a VGPR but were MS negative with oligoclonal spectra, a minimum depth of response of a VGPR would seem an appropriate starting point for patients with multiple myeloma. Due to the rarity of FLC-MS CR in patients with AL amyloidosis achieving <VGPR, limiting testing to patients who have achieved at least an amyloid VGPR would help rationalise testing to patients in whom the additional sensitivity offered by FLC-MS may provide additional prognostically useful information.

In conclusion, the results of this work support the encouraging potential of intact light chain MALDI-TOF MS based assays for response assessments of patients with PCD. At least initially, MS-based response assessments are likely to be substantially more expensive than electrophoretic-based assays. The increased cost of MS testing is

likely to be one of the main barriers to widespread adoption of MS-based assays at the current time and further studies should evaluate whether modifications, such as only performing immune enrichment for the involved specificities in follow-up samples, can be made to reduce the test of MS testing in patients with PCD. Further work should also be done to guide which patients cohorts may benefit most from MS-based monitoring and the optimal timing to perform MS-based response assessments to help rationalise the use of these more sensitive but more expensive tests for monitoring monoclonal proteins.

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