Review Article

The Paraprotein – an Enduring Biomarker

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*Jill Tate was the AACB Roman Lecturer for 2017.

Abstract
The 'paraprotein', also known as M-protein, monoclonal protein and monoclonal component, has stood the test of time as the key biomarker in monoclonal gammopathies. It continues to reinvent itself as new electrophoretic and immunoassay methods are developed that are analytically more sensitive. Use of the serum free light chain immunoassay in particular has led to new clinical discoveries and improvements in the diagnosis and monitoring of patients with plasma cell dyscrasia and other monoclonal gammopathies. In addition, minimal residual disease can be detected using mass spectrometry and flow cytometry methods.

Introduction

Approximately 40 years ago, in 1978, Miss Margaret Coles, who led the Proteins Laboratory at the Institute of Medical and Veterinary Science in South Australia, together with Dr Paul Carter, undertook an Australian tour teaching protein electrophoresis to laboratory scientists. Over the next 20 years new protein electrophoretic methods were developed. However, it was not until 2001 with the discovery that monoclonal proteins, namely monoclonal free light chains (FLC), could be detected in nonsecretory myeloma (NSMM) that measurement of the paraprotein was rejuvenated. The following review describes some of the history of monoclonal gammopathies, the importance of the measurand 'paraprotein', appropriate selection of laboratory testing according to clinical guidelines including use of serum FLC, different protein electrophoretic methods used by laboratories, and the need for more harmonised quantification and reporting of small paraproteins with the introduction of the electronic health record (eHR).

History of Monoclonal Gammopathies

Monoclonal gammopathies are a group of disorders ranging from the benign (pre-malignant) to the malignant plasma cell dyscrasias (PCD) to the lymphoproliferative disorders, e.g. benign monoclonal gammopathy of undetermined significance (MGUS), smouldering myeloma to symptomatic multiple myeloma (MM), AL (light-chain) amyloidosis, Waldenström macroglobulinaemia (WM) and plasmacytoma (**Table 1**).[1] Typically, 98% of monoclonal gammopathies produce a paraprotein that can be detected as a discrete band on protein electrophoresis of serum or urine. The paraprotein may be produced in only small amounts of a few mg/L (trace) in low tumour burden, oligosecretory PCD such as AL amyloidosis and light chain deposition disease (LCDD) disorders, compared to a concentration of over 100 g/L in large tumour burden gammopathies such as MM, WM and plasma cell leukaemia.

An additional classification, monoclonal gammopathy of renal significance (MGRS), has been recently introduced.[2,3] It is associated with kidney disease but does not meet the definition of symptomatic multiple myeloma or malignant lymphoma. MGRS was introduced to distinguish the associated paraprotein as a nephrotoxic protein independent of clonal size that may cause progressive kidney disease despite no increase in the paraprotein concentration. Treatment of this low tumour burden disease with cytotoxic agents leads to preservation of renal function as the primary goal.

Clinical laboratories performing protein electrophoresis require a range of strategies to detect both low and high paraprotein concentrations. Urine protein testing (urine total protein and electrophoresis) is important to differentiate glomerular proteinuria (that occurs in AL amyloidosis and LCDD) from the tubular proteinuria that occurs in cast nephropathy (e.g. light chain MM). Immunofixation (IFE) is important to detect small amounts (<1 g/L) of paraprotein.[3]

Table 1. Frequency of monoclonal gammopathy cases at Mayo Clinic between 1960 and 2017 (adapted from ref. 1 with an update kindly provided by Dr Robert Kyle, personal communication).

Monoclonal gammopathy	No. of cases	% of cases	Malignant high tumour burden	Malignant low tumour burden	Pre-malignant
Monoclonal gammopathy of undetermined significance (MGUS)*	32175	57.1			X
Multiple myeloma	10112	17.9	X		
Primary (AL) amyloidosis	5286	9.4		X	
Smouldering myeloma	2128	3.8		X	
Lymphoproliferative disease	1507	2.7	X		
Waldenström macroglobulinaemia**	1622	2.9	X		
Plasmacytoma	975	1.7	X		
POEMS syndrome	335	0.6		X	
Light chain deposition disease	188	0.3		X	
Plasma cell leukaemia	139	0.2	X		
Cold agglutinin disease	131	0.2		X	
Acquired Fanconi syndrome	51	0.1		X	
Scleromyxedema	39	0.1		X	
Heavy-chain diseases	46	0.1	X		
Capillary leak syndrome	50	0.1		X	
Other monoclonal gammopathies	1607	2.8			
Total number of cases	56391				

* includes Light chain MGUS; ** includes Smouldering Waldenström macroglobulinaemia
POEMS – polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes

Early History of Multiple Myeloma

The following information comes from an historical review of the discovery of MM by one of the doyens of MGUS, Robert Kyle of the Mayo Clinic.[4] The first well-documented case of MM in 1844 was of Sarah Newbury, aged 39, who broke both femurs, her right humerus, right radius, ulna and both clavicles by the time of her death. Treatment was an infusion of orange peel and rhubarb pill, in addition to opiates. Post mortem revealed that her bones had been replaced by a 'tumour' of the bone marrow (myelo) and it was called myeloma. As the disease occurred in multiple sites it was called 'multiple myeloma', although this term was not applied until 1873 by von Rustizky.[5]

The next patient with myeloma, Thomas McBean, was more extensively described and his general practitioner, Dr Watson, and Harley Street consultant Dr Macintyre noted that the patient's 'body linen was stiffened by his urine despite the absence of a urethral discharge'. Dr Henry Bence Jones, a chemical pathologist, analysed urine specimens received from both Watson and Macintyre and corroborated Macintyre's finding that the addition of nitric acid formed a precipitate which redissolved on heating and reformed on cooling. He calculated that Mr McBean was excreting more than 60 g/24 h of the protein that later became known as Bence Jones protein (BJP).[6] Indeed, the paraprotein has a long history starting with the identification of BJP by Henry Bence Jones in 1847,[7] followed by identification of its properties over the next 117 years (**Table 2**).[8-12]

Treatments – Myeloma Management in 2018

Whereas in 1990 therapeutic options for myeloma were limited to melphalan and prednisolone as the standard therapy, in 2018 multiple therapeutic options have become available including:[13]

- conventional chemotherapy
- autologous and allogeneic stem cell transplantation
- immunomodulatory drugs (thalidomide, lenalidomide, pomalidomide)
- proteasome inhibitors (bortezomib, carfilzomib, ixazomib)
- panobinostat
- monoclonal antibodies (daratumumab, elotuzumab, isatuximab)

Table 2. Properties of Bence Jones protein.

Year of discovery	Discovery	Scientists responsible for discovery
1846 and 1847	Solubility properties of BJP in urine on heating and cooling	Henry Bence Jones (who emphasised its importance in MM)[6,7]
1955	Synthesis of BJP was independent of the synthesis of the abnormal serum globulin (Ig paraprotein)	Putnam & Hardy[8]
1956	Antisera to BJP also reacted with myeloma proteins Two classes of BJP designated 'kappa' and 'lambda' were named after two scientists	Korngold & Lipari[9]
1962	Amino acid composition of the L-chain of IgG monoclonal protein and BJP from the same patient's urine were identical and had the same physical and chemical properties	Edelman & Gally[10]
1965 and 1966	BJP related to L-chain of g-globulin and each L-chain was divided into a 'variable' or V region and a 'constant' or C region, which accounts for the heterogeneity of normal g-globulins, and for the specificity and diversity of antibodies	Hilschmann & Craig[11] Titani, Whitley Jr & Putnam[12]

BJP, Bence Jones protein; Ig, immunoglobulin; L-chain, light chain

- plitidepsin
- bispecific T cell engager (BiTE®) antibodies and chimeric antigen receptor (CAR) T cells
- multiple others under investigation

As a result of the availability of new drugs, response rates and survival have increased although MM is still not a curable disease (**Table 3**). These newer therapies for MM increase overall survival (OS) when compared with conventional therapy, not only in clinical trials but in population studies as well, as confirmed by data from Sweden.[14] As a result of the use of newer treatments leading to patients living longer, the requirement for more frequent monthly protein electrophoresis testing to assess treatment response, and with the general population now living longer, more serum protein electrophoresis (SPEP) requests are being routinely ordered, with the annual number of SPEP tests continuing to increase in clinical laboratories. In the 2-year period 2015–2016, the number of SPEP requests received by the central laboratory servicing most of public pathology in Queensland increased by 10.7% (N = 22,050 in 2016) and the number of serum FLC tests increased by 31.6% (to 14,544 in the same year) in keeping with changes to clinical guidelines. In contrast, the number of random urines received for BJP testing stayed around 4200 with a 12.4% decrease in timed urines received for BJP testing (635 in 2016).

Biochemistry: the Measurand

What is a paraprotein and how is it defined? Dictionary definitions are not uniform in their descriptions of the word paraprotein and range from 'a normal or abnormal plasma protein appearing in large quantities as a result of some pathologic condition', with the term 'M component' frequently used, to 'a monoclonal immunoglobulin of the blood plasma, produced by a clone of plasma cells arising from the abnormal rapid multiplication of a single cell'. However, the majority of paraproteins in premalignant monoclonal gammopathies such as MGUS are not pathologic whereas monoclonal

Table 3. Response rates in myeloma (refs 104–109).

Regimen	Complete remission	1 year Overall Survival
Melphalan + prednisolone (MP)	2%	70%
Autologous stem cell transplantation	40%	80%
MP + thalidomide	16%	90%
MP + bortezomib	32%	92%
MP + lenalidomide	10%	~87%
Lenalidomide + dexamethasone	15%	94%
MP + bortezomib + daratumumab	43%	~90%

immunoglobulins can occur in trace concentrations and be lethal. Not only plasma cells but lymphocytic cells can over-proliferate in lymphoproliferative disorders such as in WM and other lymphomas.

Paraproteins present as a range of different proteins and may consist of an intact immunoglobulin, free light chains or heavy chains, or their fragments, produced by a plasma cell or a lymphoid cell that is proliferating abnormally. Importantly, they usually present as a monoclonal band on protein electrophoresis of serum or urine. In the case of monoclonal FLC (urine or serum BJP), there is large charge and size heterogeneity. The isoelectric point of monoclonal free light chains can range from a pH of ~4.5 to 8.5 and size may vary from 22 kDa (monomer) to 44 kDa (dimer) and higher, depending on the degree of polymerisation or aggregation, or smaller in AL amyloidosis.[15]

AL amyloidosis is an oligosecretory disease caused by a small plasma cell clone secreting light chains (ratio of λ to κ light chains, 4:1) that have abnormal folding properties. The misfolded light chains aggregate to form amyloid fibrils which are toxic for cells and tissues, depositing in vital organs. The monoclonal FLC may be low molecular mass fragments of 5–18 kDa representing the amino-terminal molecule in amyloid fibrils.[16]

Monoclonal Gammopathies – the Total Testing Process

Laboratory testing for monoclonal gammopathies, as for any clinical condition, is guided by the total testing process.[17] This comprises five separate phases involving the initial 'pre-pre-analytical phase' of the test request with advice on the most appropriate testing being provided by clinical guidelines. Next comes the 'pre-analytical phase' involving correct sample types and transport and handling of samples. This is followed by the important 'analytical phase' with optimal, state-of-the-art tests that are sensitive and/or specific for the disease being investigated. Next is the 'post-analytical phase' comprising reporting of results and their reference intervals (harmonised limits being optimal for the same methods in use) or decision limits, common units, these often being Système International (SI) to avoid errors in interpretation, and data compilation within a location (e.g. state, nation). The final 'post-post-analytical phase' refers to the same clinical interpretation of a patient's results by a pathologist, clinician or patient regardless of the method used.

Pre-Pre-Analytical Phase – Clinical Guidelines

Clinical guidelines provide guidance on the laboratory testing required for MM (**Table 4**), AL amyloidosis (**Table 5**), and WM (**Table 6**) for diagnosis, monitoring and prognostication of these monoclonal gammopathies.

Multiple Myeloma

The International Myeloma Working Group (IMWG) has its roots in the International Myeloma Foundation Scientific Advisory Board (IMFSAB) which was formed in 1995. The first IMWG publication was the myeloma management guidelines in 2003,[18] followed in 2006 by the first international uniform response criteria for myeloma,[19] and by the use of

Table 4. Protein electrophoresis, serum free light chains and response criteria for multiple myeloma.[25]

Response	Response criteria	Comment
Stringent Complete Response (sCR)	CR + normal serum FLC ratio and absence of clonal plasma cells in bone marrow	Only validated for Freelite® serum free light chain assay
Complete Response (CR)	Negative IFE of the serum and urine <5% plasma cells in bone marrow	IFE required if paraprotein not visible by electrophoresis
Very Good Partial Response (VGPR)	Serum or urine paraprotein only detectable by IFE, or ≥90% reduction in serum paraprotein plus urine BJP <100 mg/day	IFE required if paraprotein not visible by electrophoresis
Partial Response (PR)	≥50% reduction of serum paraprotein, or reduction of urine BJP by ≥90% or to <200 mg/day FLC only used if paraprotein not measurable by SPEP or UPEP	Allow use of quantitative immunoglobulin levels in patients in whom the paraprotein measurements are unreliable (e.g. IgA paraproteins co-migrating with the beta region)
Minimal Response (MR)	≥25% reduction of serum paraprotein or ≥50% reduction in urine BJP	

FLC, free light chain; IFE, immunofixation; BJP, Bence Jones protein; SPEP, serum protein electrophoresis; UPEP, urine protein electrophoresis

Table 5. Protein electrophoresis, serum free light chains and response criteria for AL amyloidosis (ref. 26).

Response	Response criteria	Comment
Complete Response (CR)	Normalisation of serum FLC levels and ratio Negative IFE of the serum and urine	IFE required if paraprotein not visible by electrophoresis
Very Good Partial Response (VGPR)	Reduction in dFLC to <40 mg/L	Only validated for Freelite® serum FLC assay
Partial Response (PR)	≥50% reduction in dFLC	Irrespective of degree of reduction in paraprotein
No response	Less than a PR	

FLC, free light chain; IFE, immunofixation; dFLC, difference in serum FLC (involved minus uninvolved FLC)

serum FLC analysis in myeloma published in 2009.[20] In 2011 three consensus guidelines were published covering the reporting of clinical trials, risk stratification and investigative work up,[21-23] followed in 2014 by the updated criteria for the diagnosis of MM.[24] The 2011 guidelines include the requirement for both normal FLC ratio and absence of clonal plasma cells in bone marrow in the IMWG new response category of stringent complete response (sCR).

The 2014 guideline was introduced to better risk stratify MM including smouldering myeloma which does not have 'CRAB' criteria. The 'CRAB' criteria that refer to end organ dysfunction are:
C: calcium elevation (>2.75 mmol/L)
R: renal dysfunction (creatinine >173 μmol/L)
A: anaemia (haemoglobin <100 g/L)
B: bone disease (lytic lesions or osteoporosis)

However, to detect the 10% of high-risk smouldering MM that progress to MM within the first two years of diagnosis, the Biomarkers of Malignancy (BOM) were introduced, namely:

- bone marrow plasma cells >60%
- involved:uninvolved serum FLC ratio >100 (using Freelite® assay). Minimum concentration of involved light chain (kappa or lambda) should be ≥100 mg/L
- >1 focal lesion on magnetic resonance imaging

The IMWG response criteria now include assessment of minimal residual disease by next generation flow cytometry, next generation sequencing and CT/PET assessment of bone lesions.[25] However, serum and urine protein electrophoresis, IFE, serum FLC and the use of immunonephelometric or turbidimetric immunoglobulin assays for paraproteins overlapping other normal proteins, especially in the beta region on SPEP, are the main protein assays performed in chemistry laboratories (Table 4).

AL Amyloidosis

Serum FLC studies and serum and urine IFE are essential in the diagnosis of AL amyloidosis and, in conjunction with bone marrow immunohistochemistry (CD138, kappa and lambda), can establish clonality. A difference between the involved FLC (iFLC) and uninvolved FLC (uFLC) of 50 mg/L was defined as assessable or measurable for response (Table

Table 6. Protein electrophoresis and response criteria for Waldenström macroglobulinaemia (ref. 29).*

Response	Response criteria	Comment
Complete Response (CR)	Negative IFE of the serum Normal serum IgM level	IFE required if paraprotein not visible by electrophoresis
Very Good Partial Response (VGPR)	≥90% reduction in serum IgM Paraprotein	Can be densitometry of SPEP or nephelometry
Partial response (PR)	≥50% reduction of serum IgM Paraprotein	Can be densitometry of SPEP or nephelometry
Minimal Response (MR)	≥25% reduction of serum IgM Paraprotein	Can be densitometry of SPEP or nephelometry
Progressive Disease (PD)	≥25% increase in of serum IgM paraprotein from nadir	Can be densitometry of SPEP or nephelometry

* It is crucial that sequential response assessments in individual patients are performed in the same laboratory using the same methodology.
IFE, immunofixation; SPEP, serum protein electrophoresis

5).[26] The highest sensitivity screening panel for detection of a paraprotein in AL amyloidosis consists of SPEP, urine protein electrophoresis (UPEP), IFE and serum FLC assays. In MGUS, with an abnormal FLC ratio, it is important to test for cardiac and renal amyloid.[27]

Waldenström Macroglobulinaemia

WM is a malignant lymphoplasmacytic disease that presents with monoclonal IgM.[1] An increasing concentration of monoclonal IgM may be associated with an increasing viscosity of the blood. Hence it is important for laboratories to be alert to the presence of cryoglobulinaemia in WM patient serum. Importantly, the response criteria guidelines recommend that sequential response assessments in individual patients are performed in the same laboratory using the same methodology. This can be either by use of SPEP or immunonephelometry.[28,29]

Diagnostic Sensitivity of Protein Testing

In a study of monoclonal gammopathies tested at the Mayo Clinic, various combinations of tests together with single tests were compared for their sensitivity of detection of true positive disease. Sensitivities of 100%, 100%, and 99.5% were achieved for MM, WM and smouldering MM, respectively, using the combination of SPEP and serum FLC whereas 88.7% sensitivity was obtained for MGUS using these same tests. The addition of IFE optimised sensitivity for detection of plasmacytoma and POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes) syndrome (89.7% and 96.8%, respectively), whereas all five tests, including urine and serum IFE, gave optimal sensitivities of 98.1% and 83.3% for AL amyloidosis and LCDD, respectively. The Mayo's screening population was limited to patients with documented monoclonal gammopathies rather than the general population with suspected monoclonal gammopathy. Whether such proposed screening panels will prove useful in terms of sensitivity and specificity in the general population needs to be assessed.[1,30,31]

Although new tests such as next generation flow cytometry and molecular sequencing, bone turnover markers and bone lesion imaging are being investigated, SPEP, UPEP, IFE and serum FLC assays remain the backbone of testing in the routine protein laboratory for the diagnosis and monitoring of monoclonal gammopathies.

Pre-Analytical Phase – Sample Type and Collection

Serum is the specimen of choice for SPEP, IFE, quantitative Ig, serum FLC and cryoglobulin quantification and isotyping (**Table 7**). Cryoglobulin is easily lost if the serum sample is not collected properly at 37 °C followed by handling at 37 °C (clotting, centrifugation, storage) to prevent loss of the protein (monoclonal and/or polyclonal) during sample preparation and analysis. It is recommended for urine protein electrophoresis and BJP quantification to use an early morning or 24-hour urine sample.

Analytical Phase

Improved analysis methods for protein electrophoresis and separation of globulins, detection of cryoglobulin, isotyping

Table 7. Specimen types preferred for protein electrophoresis and other associated methods.

Test	Sample type	Comment
Serum protein electrophoresis and IFE	Serum preferred	Plasma contains fibrinogen that can obscure a paraprotein and not allow its accurate quantification; fibrinogen can cross-react with serum FLC antisera on IFE
Serum FLC	Serum preferred	
Urine protein electrophoresis and IFE (testing for BJP) and including urine total protein and urine creatinine	Random urine (1st morning void) or 24-hour urine BJP is detected down to 20 mg/L without pre-concentration of urine, and down to 10 mg/L with pre-concentration	Monoclonal FLC in urine (BJP) should not be measured by immunoassay as values are overestimated due to the presence of polyclonal FLC
Immunoglobulins	Serum (preferred) or plasma	Also see cryoglobulin
Cryoglobulin	Serum collected and handled at 37 °C	Loss of cryoglobulin if sample collection and handling are not done at 37 °C and any precipitated cryoglobulin is not resolubilised prior to sample analysis

IFE, immunofixation; FLC, free light chain; BJP, Bence Jones protein

and the immunoassay measurement of serum FLC have contributed to improvements in the quantification and identification of small yet sinister paraproteins and minimal residual disease (MRD). The chronological development of protein electrophoresis methods is shown in **Table 8**.[32-44] The system used for quantitative protein electrophoresis of serum and urine should have high resolution and be able to detect small monoclonal proteins that may co-migrate with normal proteins, especially in the beta and alpha-2 regions.

The new technique 'monoclonal immunoglobulin Rapid Accurate Mass Measurement' (miRAMM) can detect residual paraprotein at 5 mg/L concentration by the mass spectrometry method, microflow liquid chromatography/electrospray ionisation/quadropole time of flight mass spectrometry.[44] The order of magnitude of detection is 10-fold lower than by IFE. Disease response that is negative by IFE and has a normal serum FLC can show MRD. Currently the technique is impractical for screening large numbers of samples but research is underway to develop the technique for use in routine laboratories.

Serum Free Light Chains

History and Utility in Clinical Guidelines

Measurement of serum FLC came into routine clinical laboratories following the publication in 2001 describing the presence of monoclonal FLC in 19 of 28 NSMM patients at diagnosis.[45] Use of the assay has grown since that time as FLC have been shown to be an increasingly important complementary test especially where standard SPEP, UPEP and IFE are inadequate, such as in AL amyloidosis and in NSMM or oligosecretory myeloma.[46]

The FLC assay is now well-established in the guidelines for diagnosis and monitoring of PCD.[20] According to the latest IMWG[25] and Australian Myeloma guidelines,[47] the FLC assay is recommended in all newly-diagnosed patients with PCD. Serum FLC measurement does not negate the need for 24-

Table 8. Protein electrophoresis and other methods to identify monoclonal proteins.

Year	Discovery	Scientists responsible for discoveries
1930	Moving-boundary electrophoresis; Serum globulin homogeneity	Tiselius[32]
1933	Cryoglobulin	Wintrobe and Buell[33]
1937 and 1939	Separation of serum globulins into α-, β- and γ-globulins Antibody activity to γ-globulin fraction of plasma protein Electrophoresis apparatus was 20 ft long, 5 ft high and a single electrophoresis took a full day's work	Tiselius and Kabat[34]
1950s and 1960s	Use of filter paper electrophoresis and staining with dyes Cellulose acetate replaced filter paper and protein electrophoresis became popular in clinical laboratories	Kunkel and Tiselius[35] Kohn[36]
1955	IEP for isotyping of paraprotein	Grabar and Williams[37]
1961	Concept of monoclonal vs polyclonal gammopathies	Waldenström[38]
1973	High resolution electrophoresis on agarose gel	Laurell[39]
1979 and 1980s	IFE Detection of small monoclonal bands including L-chains	Jeppsson, Laurell, Franzén[40] Whicher, Hawkins, Higginson[41]
1980s and 1990s	Capillary zone electrophoresis was becoming popular Isoelectric focusing and more advanced electrophoretic methods were popularised and introduced into Australian clinical labs	Melbourne School of Protein Electrophoresis held AACB workshops in September 1985 and November 1989[42]
2001	Serum free light chains immunoassay was introduced	Bradwell[43]
2004 and later	Mass spectrometry – 'monoclonal immunoglobulin Rapid Accurate Mass Measurement' (miRAMM)	Mills, Barnidge, Murray[44]

IEP, immunoelectrophoresis; IFE, immunofixation electrophoresis; L-chains, light chains

hour urine studies. In addition to screening, testing FLC may be particularly useful in patients with solitary plasmacytoma, smouldering myeloma and MGUS due to prognostic significance in these monoclonal gammopathies.

The response criteria were developed and validated in clinical studies in which serum FLC were measured by the Freelite® assay. Serum FLC measurement is particularly important for the monitoring of patients with light chain myeloma (LCMM), so called 'oligosecretory' myeloma where the disease is not deemed measurable by SPEP, 'non-secretory' myeloma where SPEP and UPEP are normal but an abnormal FLC is present,[21] and in AL amyloidosis.[26] Laboratories should ensure that their report contains enough data that these responses can be easily calculated, particularly the achievement of complete and very good partial remissions. Because the response categories require reference to other clinical (e.g. presence of extramedullary plasmacytomas), biochemical (e.g. hypercalcaemia) and haematological (e.g. bone marrow plasmacytosis and plasma cell clonality) data, it is not appropriate to report response categories based on protein electrophoresis and serum FLC results alone.

In 1969 Hobbs reported on BJP escape and the need to monitor urine during treatment.[48,49] More recent studies show that serum FLC is a sensitive test to detect light chain escape (LCE) in intact immunoglobulin myeloma. Light chain escape is the emergence of a de-differentiated clone of FLC-only-producing plasma cells during 'escape from treatment'.[50,51] It has been identified in patients who produce a monoclonal intact immunoglobulin at presentation, but relapse with rising production of monoclonal FLC in the serum and urine and stable or falling intact immunoglobulin paraprotein serum concentrations. In a large study of disease progression in myeloma, Zamarin *et al*. report that of the 64–71% of patients with intact Ig paraproteins and FLC at diagnosis, 11–37% relapse with FLC only.[52]

Information for Clinicians

With the introduction of three further serum FLC assays into the marketplace (**Table 9**),[53-63] the following information is recommended for clinicians:

- Present data indicate that results of various serum FLC assays cannot be used interchangeably, especially in monitoring response to therapy. Results must be interpreted in combination with serum and urine protein electrophoresis.
- An individual patient may or may not meet certain diagnostic, prognostic or response criteria, depending on the FLC assay and platform used.[15] Guidelines using serum FLC are based on the Freelite® assay.
- All assays will have limitations and may 'miss' occasional patients. Continue to use urinary BJP for screening in difficult cases.[64]
- N Latex®, Seralite® and Sebia results may read lower than Freelite® values in patients with myeloma kidney.
- Assay validation in one clinical group of patients does not necessarily imply validity in all groups of patients. Clinical validation studies are limited using N Latex®, Seralite® and Sebia FLC assays (Table 9). Freelite® and N-Latex have similar diagnostic sensitivities for AL amyloidosis when used in combination with serum and urine IFE.[65-67] Data are required to validate the use of Seralite® and Sebia assays in AL amyloidosis.
- There remains an urgent need to determine uniform response criteria for serum FLC that are applicable to all assays; e.g. in complete response (CR) would normalised FLC ratio or normalised difference in FLC (dFLC) be a more suitable parameter?
- Different diagnostic ranges for κ/λ ratio using Freelite® and Sebia assays (but not N Latex® or Seralite®) are required for end-stage renal failure (ESRF) patients.[54,58,63,68]
- It is recommended that patients be tested at the same laboratory for serum FLC measurement. This will provide a more consistent approach to monitoring disease response using the same assay and the same analyser and reduce unwarranted variation and misinterpretation of results.

Serum FLC Assay Properties

The serum κ and λ FLC immunoassays have properties that both clinicians and laboratories have become aware of since the introduction of the assays. Some of these properties are unique to specific manufacturers' anti-FLC antisera.[69] In particular, assays may vary in their antibody reactivity, antigen excess, nonlinearity and overreaction due to the presence of FLC polymers (**Table 10**).[56,59,70-75] The FLC antibodies must recognise only the epitopes which are hidden in intact immunoglobulin and exposed on FLC and there must be no cross-reaction with intact immunoglobulin. Polyclonal anti-human-FLC antisera are prepared by immunising rabbits or sheep with a cocktail of BJPs, and adsorbing the product with IgG or Cohn fraction II to remove antibodies that react with bound immunoglobulin light chains. Ideally, polyclonal anti-human FLC antibodies target the constant domain of the light chains (CL) which has little structural variation, and have adequate specificity and affinity to bind to individual monoclonal FLC. Monoclonal antibody-based FLC methods require antibodies to be directed to the CL domains present in Cκ allotypes and Cλ isotypes on the FLC and to have equivalent immunoreactivity for all variable region subgroups.

Table 9. Current serum free light chain assays and manufacturers' specifications.

Assay	Antibody (anti-κ + λ)	Method	Platform(s)	Clinical validations	Reference interval(s)	Precision: CV (%) over measuring range
Freelite® (Binding Site Group)	Polyclonal antibodies (sheep): latex conjugates	INA, ITA	Protein and multiple automated (antigen excess testing is not available on all platforms)	AL amyloidosis, MM (including NSMM, LCMM, intact Ig MM), AKI, ESRF, WM, CLL, polyclonal immunoglobulin disorders	k FLC 3.3–19.4 mg/L l FLC 5.7–26.3 mg/L FLC ratio 0.26–1.65[53] FLC ratio 0.37–3.1 (ESRF)[54] Involved FLC >500 mg/L (AKI)[55]	<20%[56]
N-Latex (Siemens)	Monoclonal antibodies: polystyrene-conjugates	INA	BN Systems (antigen excess testing provided)	AL amyloidosis, MM (including NSMM, LCMM, intact Ig MM), ESRF	k FLC 6.7–22.4 mg/L l FLC 8.3–27.0 mg/L FLC ratio 0.31–1.56[57] k/l FLC ratio 0.31–1.56 (ESRF)[58]	<5–7%[59]
Seralite and ELISA (Abingdon)	Monoclonal antibodies: gold-labelled	1. Competitive inhibition 2. ELISA	1. Cartridge & reader device (lateral flow technology) 2. ELISA	MM (including NSMM, LCMM, intact Ig MM), AKI	k FLC 5.2–22.7 mg/L l FLC 4.0–25.1 mg/L FLC ratio 0.5–2.5[59] FLC ratio 0.5–2.5 (ESRF)[60] FLC ratio 0.14–2.02; dFLC ≥400 mg/L (AKI)[61]	9–12% (2 batches of cartridge, 60 devices, 3 operators)[59]
SebiaFLC (Sebia)	Polyclonal antibodies (rabbit): latex-enhanced	ELISA	ELISA (AP22 ELITE); Assay can be adapted to other ELISA platforms	MM	k FLC 5.2–15.3 mg/L l FLC 8.2–18.1 mg/L FLC ratio 0.37–1.44[62] FLC ratio 0.46–2.23 (ESRF)[63]	k FLC: 1.9–7.6% (between day) l FLC: 6.0–6.3% (between day)[62]

AKI, acute kidney injury; CLL, chronic lymphatic leukaemia; ESRF, end-stage renal failure; FLC ratio, kappa/lambda free light chain ratio; INA, immunonephelometric assay; ITA, immunoturbidimetric assay; intact Ig MM, intact immunoglobulin myeloma; LCMM, light chain myeloma; MM, multiple myeloma; NSMM, nonsecretory myeloma; WM, Waldenström macroglobulinaemia

Serum FLC Recommendations for Laboratories

In May 2014 the Royal College of Pathologists of Australasia Immunology Quality Assurance Working Party (RCPAQAP WP) disseminated recommendations for serum FLC measurement in routine laboratories. This followed the presence of large discrepancies in serum FLC values between laboratories that were largely due to differences in sample dilutions used. The main recommendations for FLC measurement in routine laboratories are summarised below with more detailed practical laboratory information and examples of FLC measurement provided in the document at the RCPAQAP website.[76]

FLC assay imprecision goal:

- The WP recommends that a laboratory should use a serum-based control either within the reference interval or close to FLC upper reference limit values to monitor assay imprecision and any reagent lot-to-lot variation. Alternately, repeat several samples assayed with the previous reagent lot.
- Manufacturers' FLC quality controls matched to specific kit lots should be within ±20% CV of the quoted values.

Reference intervals and diagnostic range for serum FLC:

- The WP recommends using the manufacturers' κ and λ FLC reference intervals and κ/λ ratio diagnostic ranges

Table 10. Serum free light chain immunoassay properties.

Property	Comment
Precision and lot-to-lot variability of reagent	Variability between reagent lots should be checked by repeat sample assay or use of a normal/elevated serum-based control.[56,59,70,71]
Antibody specificity	Cross reactivity with bound light chains and intact immunoglobulin should be checked to avoid overestimation of serum FLC. False negative results occur due to limited reactivity (conformation of FLC) to anti-FLC antibody or because of a lack of antigen recognition (large monoclonal FLC diversity).[56,72]
Calibration traceability	No reference measurement system (reference method or reference material) is available for serum FLC. Preliminary work suggests FLC agree more closely with serum protein electrophoresis values than with immunoassay values.[73] Despite some traceability of assay calibrators to the predicate assay (Freelite®) polyclonal calibrants, absolute FLC values and ratios may differ between manufacturers' assays.[15,43,70]
Antigen excess	Serum FLC measurement spans the concentration range from ~1 to 10,000–20,000 mg/L with the potential for antigen excess to occur in 2-site sandwich immunoassays and underestimation of FLC concentration.[69]
Nonlinearity	Nonlinearity may occur at relatively low FLC concentrations due to limited reactivity with antibody or blockage of reactive sites on the FLC molecule by other proteins.[59,74]
Nephelometric overestimation of FLC	Multimers may be present and over-react in immunonephelometric assays e.g. NSMM with a 1.5–3.5-fold overestimation compared to other MM sera.[75] They are artefactual but, despite this, FLC can be used to monitor disease response.
Gap effect	At the lower and upper extremes of the Freelite® measuring range serum FLC underestimation occurs resulting in inaccurate FLC ratio.[59]
Differences in absolute FLC values and ratios between assays	Absolute FLC values and ratios may differ between manufacturers' assays and between different platforms (using Freelite® assay).[56] FLC ratios may be inaccurate at very low, suppressed FLC concentrations. It is suggested to use 'difference in FLC' (dFLC) which is involved FLC minus uninvolved FLC to monitor disease response.

FLC, free light chain(s); MM, multiple myeloma; NSMM, nonsecretory myeloma.

and for laboratories to validate manufacturers' values according to the CLSI document C28-A3.[77]
- In ESRF a different κ/λ ratio range needs to be applied when using the Freelite® and Sebia assays but not when using the N Latex® or Seralite® FLC assay.

Procedures for FLC sample dilutions to detect antigen excess and nonlinearity:
- The WP recommends that laboratories follow the manufacturers' dilution procedures for FLC measurement although for difficult samples it may be worthwhile to investigate further.
- Follow the manufacturer's recommended sample dilution procedures according to the platform used when testing a new patient and the sample gives a FLC concentration or ratio that does not agree with other clinical or laboratory findings, or is from a patient who has previously demonstrated antigen excess.
- For problematic samples, further sample dilutions may be helpful for interpreting results.
- 'Unusual' serum FLC results will still occur. Results should be interpreted in combination with serum and urine protein electrophoresis.
- Continue to use urinary BJP for screening in difficult cases.

Reporting of FLC results:
- The WP recommends that laboratories use the same FLC assay and the same platform when monitoring disease response. If there is a change of assay or platform, re-baseline FLC on one or two samples when monitoring disease progress in individual patients.
- Reporting of FLC concentrations in whole numbers from 0 to 100 mg/L is recommended. Values above 100 mg/L can be reported to two significant figures after rounding.
- For calculation of κ/λ ratio, initially retain individual κ or λ FLC raw values from the analyser to one decimal place. Depending on a laboratory's LIS, report κ/λ ratio of 0 to <10 to two decimal places and κ/λ ratio ≥10 to one decimal place or as a whole number.

- When one FLC value is within or below the reference interval and the other FLC value is below the reference interval, calculation of the κ/λ FLC ratio is problematic. In such instances, where imprecision is reported to be high, the WP recommends that laboratories do not report the calculated κ/λ ratio and indicate that the ratio is of uncertain clinical significance.
- The WP recommends that the assay type (Freelite®, N Latex®, Seralite®, Sebia) be mentioned in the report to avoid confusion by clinicians when patients are tested by different pathology providers.
- Serum FLC testing should be further integrated into routine clinical practice as it is a sensitive marker of disease response and light chain escape.

Laboratory education:

- Laboratories should provide an educational strategy ready for clinicians on implementation.
- Carefully review assays when results of clinical validation studies are published.

Urine BJP and Serum FLC in Monitoring Light Chain Myeloma

Clinical Studies

The current IMWG recommendation for monitoring of LCMM is to measure 24-hour excretion of BJP.[21] Serum FLC levels should only be used for response assessment when both the serum and urine M-component levels are deemed not measurable. The BJP recommendation was based on the E9486 study of FLC response compared to urine BJP response of which only 14% of the whole 399 samples had LCMM and in which there was a poor correlation between 24-hour BJP and FLC, and FLC response after 2 months did not predict progression free survival (PFS) or OS.[78] However, in the recent Intergroupe Francophone du Myélome (IFM) 2009 study of 113 LCMM patients by Dejoie *et al.*, all diagnostic samples had an abnormal κ/λ FLC ratio and all involved FLC were measurable for monitoring (≥100 mg/L).7[9] By contrast, only 64% of patients had measurable levels of monoclonal protein (≥200 mg/24 h) in UPEP.[79] In addition, during monitoring, normalisation of FLC ratio better predicted PFS and OS than if negative by urine IFE. In a further study comparing serum FLC and spot urine BJP/creatinine ratios at diagnosis in 576 LCMM from the UK Myeloma IX and XI studies, Heaney *et al*. showed that, whereas only 3 could not be monitored by serum FLC, 116 did not have measurable BJP. Further, like the IFM study, serum FLC response predicted outcome and enabled sensitive monitoring for patients.[60] Both studies confirmed that serum FLC, not urine specimens, should be used to evaluate response in LCMM. However, these findings have not yet been incorporated into IMWG criteria.

Current BJP Quantification and Reporting

The quantification of BJP by clinical laboratories is variable. The percentage of BJP detected on UPEP shows variation between laboratories due to differences in UPEP methods, percent BJP determined by densitometry and if urine has been pre-concentrated prior to electrophoresis. The variation in BJP quantification is approximately 5-fold depending on the urine total protein method and the reactivity of BJP to manufacturers' reagents.[80,81] Hence, measurement of BJP is variable and, similar to serum FLC, the same laboratory and method should be used when monitoring LCMM.

Bence Jones protein units also vary between laboratories. It is recommended in Australia to report urine total protein, BJP, and other proteins, including urine albumin, in mg/L concentration, mg/24 h excretion, and mg/mmol creatinine for protein/creatinine ratios to achieve harmonised units.[82-84] However, according to the RCPAQAP paraprotein survey conducted in early 2018, 23 participating laboratories reported BJP concentration in either mg/L (10) or g/L (13), 18 reported BJP excretion in either mg/24hr (9) or g/24hr (9), and few reported the BJP/creatinine ratio (4 laboratories in mg/mmol and 1 in g/mol).[85]

Serum FLC or BJP – Which to Use?

Both the clinical studies by Dejoie and Heaney comparing serum FLC versus BJP suggest there is little role for UPEP in monitoring LCMM in most patients.[60,79] Urine BJP quantification is poorly done and 24-hour samples are difficult to collect and measure. Serum FLC has the pragmatic advantage that it is a single serum sample that can be used also for SPEP. Note that serum FLC assays have their own problems, involving the occasional failure to react, differences in absolute values between assays, and differences in % reductions do not correlate precisely. There is still the need for laboratories to offer urine BJP screening to detect occasional patients not measureable by FLC assay.[64]

Harmonisation of Laboratory Practices

In 2012 the Australasian Association of Clinical Biochemists (AACB) together with other societies published recommendations for standardised reporting of protein electrophoresis in Australia and New Zealand.[86] Recommendations largely covered analysis, including methods of quantification, general interpretative commenting and commenting for specimens with paraproteins. Recent surveys through the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) in 2017 and the RCPAQAP in early 2018 together with local state paraprotein sample swaps, also in 2018, suggest that quantification of small bands and their reporting remain highly variable.[85,87,88]

Currently the clinical guidelines for monoclonal gammopathies do not provide any guidance on which electrophoresis methods should be used for quantification of paraproteins, especially those that overlap normal proteins in the beta and alpha-2 regions on SPEP. IFCC and RCPAQAP surveys as well as those conducted in Canada and the USA indicate there is a need for greater harmonisation of electrophoresis practices when quantifying paraproteins.[85,88-90] In the 2016 RCPAQAP program for paraprotein, the between-laboratory variation ranged from 14% CV at 33.5 g/L mean paraprotein concentration to 50% CV at 1.6 g/L; however, the range of paraprotein concentrations reported by laboratories was far wider. This effect is magnified for paraproteins that comigrate with normal serum proteins, most typically in the beta region. For example, a monoclonal beta-migrating IgA lambda paraprotein, median concentration 6.0 g/L for 53 laboratories gave a range of 2.0–15.6 g/L.[81]

Currently quantification of gamma- and beta-migrating paraproteins by the perpendicular drop (orthogonal, top to bottom) method is recommended in France despite differences in gel and capillary protein electrophoresis methods.[91] In the recent RCPAQAP survey and paraprotein sample swap, the gating method predominantly used for both gamma and beta paraproteins was the perpendicular drop method.[85,87] Alternate gating methods include corrected perpendicular and tangent skimming methods especially for beta paraproteins.[92,93]

Harmonisation of Protein Electrophoresis Reporting

The eHR is driving the demand for harmonisation in Australia. An inability to harmonise will impact on patient care and may result in misinterpretation of results leading to wrong treatments and poor patient outcomes. Harmonisation requirements for reporting of protein electrophoresis and serum FLC include units and terminology, reference intervals, reporting formats and interpretative commenting (**Table 11**).

The need for laboratory harmonisation has accelerated more recently with the introduction of the eHR, especially in Australia where patients have the right to have their blood analysed at any laboratory, not necessarily the one indicated on the test request slip. This means that if they are having their paraprotein monitored it is best to have blood tested by the same laboratory using the same method each time. Prior to this situation patients attended the same laboratory for repeat testing and it did not matter if SPEP concentrations for a patient's disease response were not exactly the same depending on the electrophoresis method used or the gating method used to quantify the paraprotein as it was the change in value that was significant.

In 2000 the AACB began a pilot program for the quality assessment of chemical pathology patient report comments.[94] Consistency of interpretative commenting is an important component especially in protein electrophoresis.[95,96] The RCPAQAP for interpretative commenting has been established to seek harmonisation in the operation of external quality assurance schemes for interpretative comments,[97] and has been extended to the international level.[98] The IFCC working group proposes a standard reporting structure based on the DIKW acronym (Data, Information, Knowledge, Wisdom).[99] In the case of interpretative comments for protein electrophoresis, the data required involves the measurements of tests stipulated in clinical guidelines; the information derived describes the pattern of the data, e.g. delta when monitoring disease response; knowledge is the application of the patient history for example; and wisdom is provided by appropriate interpretative comments that answer the clinical question and suggest additional follow-up tests if clinically indicated.[86,100]

Reporting of Small Abnormal Bands

Small abnormal bands, typically ≤1g/L, but of a different isotype or position can be seen on SPEP in patients with a known paraprotein. However, in patients without a known paraprotein, the presence of a new small band can signify important pathologies such as AL amyloidosis, oligosecretory myeloma or lymphoma but is more commonly due to infectious or inflammatory diseases. In myeloma following autologous or allogeneic stem cell transplantation or post-novel agent therapy, oligoclonal and small bands should be reported. However, the overcalling of these small bands can result in unnecessary investigations whereas not reporting these small bands may lead to delayed diagnosis of important oligosecretory PCD. These small bands need to be recognised but should not be reported as new paraproteins, i.e. terms such

Table 11. Post-analytical harmonisation of protein electrophoresis.

What can be harmonised?	1.	Units and terminology
	2.	Reference intervals
	3.	Reporting formats
	4.	Interpretative commenting
Why should we harmonise?	1.	To remove unwarranted variation
	2.	To enable result comparison between laboratories
	3.	To facilitate better patient management

Table 12. Clinician requirements of reporting of protein electrophoresis.

What do clinicians really want?	1. Is a paraprotein present? 2. How great is its concentration? 3. Cumulative reporting
What else do clinicians want?	1. Sufficient information to be able to calculate response to therapy 2. A uniform approach to reporting of paraproteins that migrate with normal serum proteins in the beta/alpha-2 fractions 3. Recognition and reporting of oligoclonal and small bands that occur post-transplant or post-monoclonal novel agent therapy 4. Consistent reporting of paraproteins on the cumulative report

as 'paraprotein' or 'monoclonal protein' should be avoided as they can be a potential source of confusion to clinicians and may suggest relapsed myeloma. In the majority of cases they are associated with improved remission depth and outcome and do not reflect relapse with isotype switch (**Table 12**). A problem for the eHR is including such small bands in the paraprotein detection field which can lead to multiple bands being viewed in cumulative reports hence causing clinician confusion. These small abnormal bands are best dealt with by appropriate commenting rather than accurate quantification.

In the case of new small bands occurring in patients with a known paraprotein, it is important that reports indicate the uncertainty of these small bands so as reduce patient and clinician anxiety and unnecessary investigations. Examples of comments are given in **Table 13**. The monoclonal antibody daratumumab is an interferent in protein electrophoresis and is present in the cathodal gamma region on SPEP. Similarly, elotuzumab can interfere and is found in the mid-gamma region. Daratumumab is a monoclonal antibody (mAb) against CD38 which is highly expressed in myeloma and also on normal lymphoid cells and is highly active as a single agent in advanced myeloma.[101] Presence of the therapeutic IgG kappa mAbs daratumumab and elotuzumab can interfere with assessment of CR in a patient with IgG kappa myeloma if the patient's band is a similar mobility. The mAbs are typically present in trace or up to 1 g/L on SPEP/IFE. The 2015 IMWG response criteria footnote states: 'Also, appearance of monoclonal IgG kappa in patients receiving mAbs should be differentiated from the therapeutic antibody.'[102] Methods such as the Daratumumab IFE reflex assay (DIRA) or the HYDRASYSTM Shift (Sebia) method can be used to identify the presence or absence of daratumumab.[103] Laboratories should know the position of mAbs on their SPEP system. An IgG kappa band of similar electrophoretic mobility to that of daratumumab (or elotuzumab) in a patient known to have IgG kappa myeloma could represent the presence of a therapeutic mAb and appropriate clinical correlation is required such as assessing clinical notes in the patient's eHR to check if on a therapeutic mAb.

Other examples of interpretative commenting in protein electrophoresis reporting can be found in the 2012 recommendations.[86] Updated comments will be available soon in a further report in an Australasian-wide effort to achieve greater harmonisation of laboratory practices in protein electrophoresis.

Acknowledgements: Thanks go to the many people who have taught me about protein electrophoresis and monoclonal

Table 13. Interpretative comments in patients with a known paraprotein and occurrence of new small bands or bands with similar mobility to therapeutic monoclonal antibodies.

Pattern	Minimal Comment
Small abnormal band of different electrophoretic mobility from original paraprotein	There is a small (type: e.g. IgG kappa) band, approximately (amount: e.g. 1 g/L) on a background of a polyclonal and/or oligoclonal pattern. This band is different from the original paraprotein. Its clinical significance is uncertain.
IgG kappa band of similar electrophoretic mobility to that of daratumumab (or elotuzumab) in a patient known to have IgG kappa myeloma	A monoclonal IgG kappa band, approximately (amount: ≤2 g/L) has been found in the gamma fraction on immunofixation. The band has similar mobility to that of daratumumab (or elotuzumab). This could represent the presence of a therapeutic monoclonal antibody. Clinical correlation is required.

gammopathies over many years including my close colleague Dr Peter Mollee (Head of Multiple Myeloma and AL Amyloidosis Clinics at Princess Alexandra Hospital), who continues to collaborate closely with the AACB, Dr Peter Hickman, who always encouraged scientific collaborations with the local Haematology Department, and the many Proteins' staff, other collaborators and RCPAQAP who I have worked with on various projects.

Competing Interests: None declared.

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