Editorial

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Protein electrophoresis and serum free light chains in the diagnosis and monitoring of plasma cell disorders: laboratory testing and current controversies

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In 1999 Keren and colleagues published laboratory recommendations for the testing of monoclonal proteins (M-protein, also known as monoclonal component or paraprotein) [1, 2]. Now, 17 years later, *Clinical Chemistry and Laboratory Medicine* (*CCLM*) is publishing a special issue devoted to laboratory testing in the diagnosis and monitoring of plasma cell disorders. This publication is long overdue – the literature in this specialized clinical laboratory area has failed to keep pace with the changing clinical guidelines. It is important that all laboratories working in this specific area keep up-to-date with best practice in laboratory testing and reporting and to be aware of current clinical guidelines in the field of protein electrophoresis and serum free light chain (FLC) measurement. Failure to do so has the potential to adversely impact on patient management.

Laboratory testing for plasma cell dyscrasias (PCD) continues to evolve and established treatment resources such as autologous stem cell transplantation and new immunomodulatory agents, proteasome inhibitors and monoclonal antibody therapies have greatly improved the outcome of patients with multiple myeloma but have also led to more complicated protein electrophoretic patterns and often difficult interpretation and reporting. This together with greater use of serum FLC measurement, in keeping with clinical guideline recommendations, is changing the way that clinical laboratories must organize and report the results of their tests.

At the same time the heavy/light chain assay and the newer technology, mass spectrometry with potentially better accuracy and greater analytical sensitivity, will offer improved ways to measure minimal residual disease and relapse. As with the introduction of any new technology, new assays will need to be validated for routine use in various clinical scenarios and for different laboratory settings in PCD. Whereas large referral laboratories may see a majority of PCD cases and studies can concentrate on diagnostic sensitivity, routine hospital laboratories are faced with populations that have diverse pathology. Testing for PCD may require different approaches to accommodate a laboratory's resources and client population, and a "one size fits all" approach may not be appropriate for all laboratories. These issues will provide the laboratory with challenges for the future.

In this special issue we are pleased to present a range of papers written by experts in the field. These experts include scientists and pathologists and, importantly, clinicians who utilize the results of the laboratory testing. The collaboration between the laboratory and the clinician should not be underestimated given the importance of interpreting and reporting results based on an understanding of the clinical context.

Section 1: Laboratory testing as recommended by the guidelines and the International Myeloma Working Group

The monoclonal gammopathies cover a wide range of disease presentations from the low tumor-burden AL amyloidosis to the premalignant monoclonal gammopathy of undetermined significance (MGUS) to the malignant multiple myeloma (MM). The presence of a monoclonal protein is associated with the majority of monoclonal gammopathies but their concentration may vary from only a few mg/L of serum monoclonal FLC to g/L of monoclonal intact immunoglobulin that may be detected in serum, urine and/or cells from bone marrow and other tissue biopsies. In addition, by virtue of the immunoglobulin gene rearrangement and somatic hypermutation, each patient's monoclonal protein has a unique chemical structure. To detect such a heterogeneous group of monoclonal proteins, it is essential that the routine laboratory uses a range of strategies.

In this special issue Willrich and Katzmann describe the latest recommended testing in their up-to-date review of laboratory requirements for the diagnosis and monitoring of MM and related PCD [3]. The International Myeloma Working Group (IMWG) recommends a screening panel of serum protein electrophoresis (SPEP), immunofixation electrophoresis (IFE), and serum FLC for diagnosis, and if AL amyloidosis is suspected, also urine protein electrophoresis (UPEP) and IFE. Quantification of the M-protein is used to monitor the plasma cell clone's response to therapy except where the M-protein overlaps other comigrating proteins or is so small that it cannot be quantitated by electrophoresis. In the case of IgA M-proteins, nephelometric quantitation of total IgA or heavy/light chain assays that measure IgA-κ and IgA-λ concentration separately can be used to monitor disease response. International guidelines for classification of myeloma response recommend use of the dFLC (difference between the involved and uninvolved FLC) in place of the M-protein concentration determined by densitometric analysis if the serum M-protein is <10 g/L or urine Bence Jones protein is <200 mg/24 h [4]. For this oligosecretory group of patients, if the FLC ratio becomes normal then IFE is required to confirm the absence of the M-protein [5]. The authors go on to discuss the need for more analytically sensitive assays, such as mass spectrometry, to detect minimal residual disease that is negative using SPEP, IFE and FLC.

Mollee and Merlini next review the guidelines for diagnosis, monitoring and prognostication of AL amyloidosis, a protein-folding disorder in which monoclonal immunoglobulin light chains or their fragments are the disease-causing toxic agent [6]. Diagnostic guidelines recommend that laboratories use the highest sensitivity screening panel consisting of SPEP, UPEP and IFE, and serum FLC by immunoassay. In MGUS with an abnormal FLC ratio, NT-pro B-type natriuretic peptide and urinary albumin are used as additional diagnostic markers to detect possible cardiac and renal amyloidosis. Current guidelines use quantitative serum FLC targets derived from the polyclonal-based FLC assay to determine disease response; it is important to note that different cut-offs are needed for other FLC assays as values cannot be used interchangeably between assays.

Recently a new entity called monoclonal gammopathy of renal significance (MGRS) was introduced [7]. Monoclonal gammopathies associated with kidney disease but not meeting the diagnostic criteria for myeloma or lymphoma are reclassified as MGRS, thus enabling these low tumor burden conditions to be treated with cytotoxic agents to reduce the toxic action of the M-protein on renal function. Leung et al. [8] describe the laboratory tests required to diagnose and monitor MGRS including SPEP, UPEP, serum IFE and FLC. Although UPEP has low sensitivity for detection of M-protein, it does provide additional information about the type of renal injury, i.e. tubular proteinuria as occurs in light chain cast nephropathy, and glomerular proteinuria as occurs in AL amyloidosis and light chain deposition disease. The authors go on to discuss the future use of more sensitive mass spectrometry techniques that will assist in detecting minimal residual disease and urinary exosomes as a renal response biomarker in MGRS.

Validation of guideline recommendations is essential to their clinical uptake. In an original paper by Palladini and colleagues [9] the performance of FLC measurement in AL amyloidosis patients with moderately impaired renal function vs. severe renal dysfunction is compared. As a part of this evaluation they show that use of a renal reference range for κ/λ FLC ratio [10] increases diagnostic sensitivity for monoclonal λ clones but reduces it for monoclonal κ clones. The study reinforces the importance of combining FLC measurement with adequately sensitive IFE of both serum and urine.

Section 2: Serum and urine protein electrophoresis and immunofixation testing

In this next group of papers and case reports on PCD, Keren and Schroeder begin by reviewing the electrophoretic and immunochemical methods that have been used over the years to quantify M-proteins in serum [11]. They explore the limitations of past and current methods and describe new methods to improve the accuracy of measurement of low-concentration M-proteins and quantify isotype-specific immunoglobulin classes, and liquid chromatography-tandem mass spectrometry to obtain more analytically sensitive measurements of residual M-protein.

A problematic area of laboratory testing for PCD concerns that of screening IFE as an initial procedure to investigate M-proteins. In his "Point" paper, Pretorius [12] puts the case to replace SPEP and UPEP with a screening IFE using a single application of antisera directed against heavy and light chains. He argues that there is no clinical threshold where small M-proteins can be dismissed as not significant and states: "… it does not logically follow that that these individuals will be investigated unnecessarily". In addition the advantages for the laboratory from screening IFE are fewer reflexive IFE procedures being performed and hence reduced costs and faster turnaround time. Smith et al. in their "Counterpoint" article [13] put the case against "quick fixes" and argue that while screening IFE may be useful when SPEP has poor resolution in the

β-region, it is of less benefit in laboratories using higher resolution gels to detect IgA M-proteins. They question the clinical significance of all small bands, many which represent either transient responses to infectious and auto-immune conditions, or very low risk MGUS. Additional costs and patient anxiety caused by regular patient follow-up in this group may also be problematic.

Another area of protein electrophoresis involving heterogeneity of laboratory practice is in reporting. The current wide variation in reporting of electrophoresis is likely to have an impact on patient safety due to misinterpretation of poor commenting. In his opinion paper, Moss [14] presents good reasons why we should move towards harmonized reporting of SPEP and UPEP by development of an intuitive commenting system tool that ideally could be supported by the vendor. He notes that such a product would be subject to periodic review to ensure there was continuing alignment with clinical guidelines.

Three case reports are then presented that illustrate the challenges of traditional laboratory testing (i.e. electrophoresis and IFE) and describe clearly the need to use more than one analytical approach for the correct management of these clinical situations. The molecular structure of the monoclonal protein is so variable that only the use of analytical techniques based on different principles can guarantee the identification and the quantification of the largest possible number of the monoclonal components.

Sečník et al. [15] present a case of AL amyloidosis in a patient with light chain multiple myeloma where the SPEP was repeatedly negative and the urine IFE result ambiguous; the serum FLC test was positive for κ light chain production. Other studies involving large numbers of patients with AL amyloidosis [16] have demonstrated that only the combined use of the traditional tests with the FLC serum measurement can ensure a near 100% identification of the monoclonal protein in AL amyloidosis, confirming thus that the two approaches (traditional testing plus FLC measurement) are complementary rather than alternative in amyloidosis.

The next two cases deal with the monitoring of patients after treatment. Henry and Glegg [17] describe the modification of the serum electrophoretic pattern of a patient with multiple myeloma following therapy: during remission the original monoclonal band was replaced by a number of small abnormal bands. The appropriate identification of the nature (oligoclonal vs. monoclonal) of these bands was possible only by using an isoelectric focusing technique. The use of this technique is an important novelty in this specific field; the related reporting to clinicians should be carried out with great care because of the potential clinical significance of biochemical relapse. The technique is not easy to perform and/or interpret, and should probably be restricted to specialized laboratories; however, it could be of great help in certain complicated cases. The third case is about a rather novel pattern of relapse known as “light chain escape” [18]; the pattern is probably induced by recent therapeutic strategies and is particularly demanding from a clinical point of view. Caldini et al. observed that FLC measurement could predict the relapse months before the Bence Jones protein determination. Use of the same test for follow-up of disease is recommended for monitoring of MM [4]. However, Zamarin et al. have illustrated that use of the FLC assay could detect relapse even in patients with measurable M-protein and that abnormal FLC was often the first indicator of relapse or progression in patients with FLC escape [19]. In the largest reported series of MM patients who relapsed with FLC escape, Brioli et al. highlighted the importance of monitoring FLC when clinical relapse is suspected [20].

Section 3: Serum free light chain methods and controversies

After the first burst of enthusiasm since the availability of the FLC test (as always happens with novelties) and the issuing of the related international recommendations [21], some analytical and clinical limitations of the immunological assay began being reported. Moreover, after the introduction of a different assay, and then the release of a third one, both based on monoclonal antibodies, it became evident that these assays show distinct characteristics. To help the *CCLM* readership in understanding the advantages and pitfalls of the available assays, this section of the issue includes two mini-reviews by Carr-Smith et al. [22] and te Velthuis et al. [23] discussing the clinical and analytical characteristics of the polyclonal (Freelite) and monoclonal (N Latex and Seralite)-based assays. These reviews are accompanied by an opinion paper by Graziani [24] summarizing the features of the different assays. Considering the peculiarity of the monoclonal proteins, optimization of the analytical aspects is difficult to achieve. In addition, the absence of an international standard hampers the identification of the method showing the best accuracy. Good-quality studies examining the analytical performances of the three methods are needed to help clinical laboratories decide which assay to use; however, clinical validation of the assays in different diseases and in different clinical settings is also of pivotal importance.

The question of accuracy of serum FLC measurement remains unresolved. Due to the wide heterogeneity of monoclonal and polyclonal free light chains, different selectivity

of assay antibodies for FLC measurement, and interferences that may be assay and/or platform-specific, differences in absolute FLC concentrations can occur between the various polyclonal and monoclonal antibody-based immunoassays and values cannot be interchanged. Jacobs et al. [25] describe how these issues can cause non-harmonized, non-equivalent results between different methods and hence the importance of interpreting FLC results in the clinical context. The authors discuss the inability to transfer the clinical guideline recommendations for FLC measurement across all assays in all patients, the clinical consequence being that certain diagnostic, prognostic, or response criteria may or may not be met, depending on the FLC assay and platform used.

An important clinical assessment is whether treatment results in a clinically significant decrease in the quantity of monoclonal intact immunoglobulin or serum FLC in PCD. Information on the biological variation in healthy subjects and in stable PCD can be used to determine a clinically significant change. Toftmann Hansen [26] describes the current status of this information and the need for further FLC studies in patients with PCD and in the elderly. These data can also be used to optimize the setting of desirable performance goals for monoclonal intact immunoglobulin and serum FLC measurement.

The two case reports in this section [27, 28] illustrate how difficult the detection and quantification of monoclonal proteins can be in specific patients, and confirm once more that a combination of techniques (including advanced diagnostics such as mass spectrometry and flow cytometry) are necessary in certain circumstances. The first case by Milani et al. [27] describes a patient with cardiac amyloidosis where the routine serum and urine IFE and serum FLC measurement were repeatedly negative and the amyloid clone could be identified only by using a high-resolution in-house urine IFE method and bone marrow flow cytometry. The assessment of the response to treatment was possible in this patient only using these sophisticated techniques. Patients with renal diseases and monoclonal gammopathies also pose a serious diagnostic challenge for the clinical laboratory. The second report by Levinson [28] describes two patients with renal insufficiency and PCD, where the serum FLC measurement failed to detect the monoclonal protein in one case and underestimated FLC in the second. The use of the traditional tests and the serum FLC measurement ultimately succeeded in resolving the diagnostic issue. The laboratory specialist is in the ideal position to resolve these difficult situations as he/she has a complete knowledge of the technical tools; if clinical information is provided in a timely manner to the laboratory, then additional targeted testing can be performed that will enhance the patient management.

The important issue of FLC measurement in patients with renal impairment has been discussed earlier in patients with AL amyloidoisis [6] and MGRS [8], and is also the subject of an original contribution in this section by Kennard et al. [29]. It is well known that a specific renal reference range for κ/λ FLC ratio is required when measuring serum FLC by the polyclonal-based Freelite method in patients with renal insufficiency [10], while this is not the case if the monoclonal-based N Latex method is used [30]. This is due to the much higher serum concentrations of λ light chain measured by the N Latex method. This study, based on a large number of patients on hemodialysis, confirms the finding; the novelty of the study is the demonstration that the discrepancy between the two methods attenuates post-dialysis. While further studies are certainly required to understand the reason for the discrepancy, the practical implication of these results (consistent over a number of independent studies) is that the clinical laboratory should clearly report a renal reference range for κ/λ FLC ratio if serum FLC is measured with the Freelite method. This is of particular relevance in the presence of oligosecretory diseases where a minimum deviation from the normal κ/λ FLC ratio is of diagnostic significance as described by Palladini et al. in this issue [9].

Section 4: New laboratory assays and challenges

As discussed in Section 2 [11], one way to improve the accuracy of measurement of low-concentration M-proteins includes use of immunoassays that quantify isotype-specific immunoglobulin classes. In their "Point" paper, Evans et al. [31] describe the advantages of using IgA-κ and IgA-λ heavy/light chain (HLC) assays as a more accurate method for quantification of monoclonal IgA proteins that overlap normal proteins in the β-region on electrophoresis. The increased sensitivity of HLC assays and use of the calculated IgA-κ/IgA-λ HLC ratio may give additional information about residual disease and predict relapse earlier when IFE is negative. IgA HLC may prove to be a suitable alternative to the combination of SPEP, IFE and total IgA measurement in response monitoring and risk stratification [32]. As with all new tests, there are arguments against blanket use of the assay as explained by Paolini in the "Counterpoint" [33]; selective use of the assay, only in certain cases, is warranted at this stage until trials show the positive impact of the immunoassay on clinical management. An example of another use of HLC assay is given in the report from Altinier et al. [34] on monitoring of patients with POEMS disease.

As in many areas of Laboratory Medicine, mass spectrometry is being used to quantify various analytes down to very low concentrations. Investigators from the Mayo Clinic have developed high resolution mass spectrometry methods that can measure monoclonal intact immunoglobulins and polyclonal light chains in serum [35, 36]. In the paper by Barnidge et al. [37], the authors describe in detail the "monoclonal immunoglobulin rapid accurate mass measurement (miRAMM)" methodology that uses microflow liquid chromatography-electrospray ionization-quadrupole-time-of-flight mass spectrometry and its application to the detection and quantification of serum FLC in AL amyloidosis. The proof of concept experiment confirmed the presence of monoclonal FLC in a majority of samples positive by FLC immunoassay and also identified large molecular weight heterogeneity of FLC in AL amyloidosis.

The novel therapeutic options now available pose serious challenges to the clinical laboratory, as illustrated in the next three papers. As a consequence, the laboratory technique should be updated to meet new needs. One of the novel therapeutic approaches is the use of humanized monoclonal antibodies. These antibodies, if present in blood above a certain concentration, can be detected as small IgG-κ bands on SPEP and IFE testing and possibly misinterpreted as disease related monoclonal bands. Willrich et al. [38] describe the electrophoretic properties of a number of therapeutically administered antibodies and the ability of miRAMM to detect very small quantities of the monoclonal antibodies, e.g. rituximab. These small bands and other post therapy, transient monoclonal and/or oligoclonal bands that are often present post immune reconstitution are considered an analytical interference and may confound the assessment of complete response (CR) in myeloma. Durie et al. [39] have recently revised the wording of the IMWG definition of CR that requires disappearance of the original M-protein associated with myeloma on IFE regardless of the presence of unrelated secondary bands including artefact due to monoclonal antibodies. Hence laboratories will need to be able to distinguish residual therapeutic antibody from the original M-protein, in particular IgG-κ bands. McCudden et al. [40] describe a new daratumumab immunofixation reflex assay (DIRA) that can distinguish the therapeutic antibody from M-protein. The authors recommend a testing algorithm that can be applied to small bands <2 g/L present post therapy. Examples illustrating the problem are shown for a number of cases in a report by van de Donk et al. [41] and highlight the clinical consequences of the possible missed recognition of the analytical interference. It needs to be emphasized once more that the laboratory specialist and the clinician should cooperate closely in exchanging information about the type and timing of antibody therapy (especially in the complicated field of the plasma cell dyscrasia), because it is the only way to improve the patient management and his/her outcome.

As laboratory and clinical practitioners in the diagnosis and monitoring of plasma cell disorders, we hope that this special issue will provide other practitioners with a broad overview of current and future laboratory testing in the field of protein electrophoresis and serum FLC measurement. There remain contentious areas of laboratory best practice that will require further clinical studies to provide the evidence for or against introduction of new tests and removal of old ones. With the introduction of new patient treatments and more sensitive technologies to detect residual disease, the laboratory must continue to adopt changes in testing practices that will add value to patient management.

As a postscript to this issue, *CCLM* recently received an interesting Letter to the Editor about the quantitative FLC differences between Freelite and N Latex monoclonal λ FLC results [42]. Using gel chromatography and Western blotting, with and without disulfide bond reduction of the sample, the authors concluded that the Freelite assay may selectively recognize the dimeric λ FLC in a patient with MM whereas the N Latex assay appeared to recognize the monomeric form. As the authors note, further studies are required to elucidate the relationship between the structure and pathological properties of monoclonal FLC.

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