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IMMUNOGLOBULINS DETECTION AS BIOLOGICAL MARKERS OF MULTIPLE MYELOMA

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ABSTRACT

Assessing serum biological markers is an essential component of detecting and monitoring multiple myeloma disease progression. Multiple myeloma is the second most common blood malignancy, and represents approximately 1% of all cancers with a disease burden of approximately 120,000 new cases worldwide per year. Multiple myeloma is typically characterized by the observation of monoclonal plasma cells, the presence of circulating monoclonal protein immunoglobulin, and related organ damage. Protein patterns can be altered in the presence of monoclonal protein (M-spike present), AAT deficiency (reduction in alpha-1 Antitrypsin), as well as nephrotic, acute phase, or inflammatory syndromes, which can result in changes in multiple fractions. Additionally, capillary zone electrophoresis multichannel techniques have been introduced that monitor migration of serum proteins by recording optical absorption at 210 nm in an optical window in the capillary. Measurement of intact Ig'κ, Ig'λ, and Ig'κ/Ig'λ ratio has been made possible with the recent availability of heavy light chain immunoassays (such as The Binding Site's Heavylite) or the intct Ig subsets: IgGκ, IgGλ and IgAκ and IgAλ, Ig'κ, Ig'λ, and Ig'κ/Ig'λ ratios. These assays utilize epitopes which span specific intact heavy and light chain pairings. The primary reason for performing serum protein electrophoresis is to discover a paraprotein or B cell dyspraxia. An irregularity in the gamma region can be due to a small monoclonal band, free light chains (FLC) or oligoclonal IgG. Other findings of clinical significance include increased alpha-1 and alpha-2 globulins indicative of an acute phase response, a decrease in alpha-1 globulins suggestive of alpha-1 antitrypsin (A1AT) deficiency (that can be followed up with phenotyping to check for a clinically significant A1AT variant), an increase in the beta-1 region suggestive of increased transferrin and iron deficiency, a polyclonal increase in gamma globulins indicative of in inflammation or infection of liver disease. Evidence of organ damage resulting from multiple myeloma clonal plasma cell proliferative disorder includes the CRAB criteria of hypocalcaemia, renal insufficiency, anemia, and bone lesions. The main reason for performing urine protein electrophoresis is to find a light chain myeloma producing an excess of free light chains (Bence Jones protein), an important part of a myeloma screen. A band in the urine protein electropherogram may also result from an intact monoclonal immunoglobulin, especially if the patient has poor renal function. Immunofixation is important in defining the nature of the band and in distinguishing between Bence Jones protein and an intact monoclonal protein originating from the serum. From the urine electropherogram we can also tell if the proteinuria is of glomerular origin with a predominance of albumin, or if it has tubular components with excretion of smaller molecular weight proteins such as retinol binding protein and alpha-1 microglobulin. Fragmented albumin in urine is occasionally seen but is of unknown significance.6 Historically, urine has been concentrated by either removal of water from the specimen leaving the proteins in higher concentration, or by centrifugation whereby the proteins are spun away from the majority of the water.

Demonstration of the protein components of urine from concentrated specimens was originally performed on cellulose acetate and later on agarose and high-resolution agarose gel. Monoclonal proteins are routinely visualized by serum protein electrophoresis (SPEP) and/or Immunofixation electrophoresis (IFE) (See Figures 1 and 2). IFE and SPEP can also be performed on urine to look for the presence of Bence Jones proteins and are often designated as UIFE and UPEP analysis. However, urinalysis protein strips are relatively insensitive to Bence Jones proteins, and absence of elevated urine protein does not exclude multiple myeloma. Immunoglobulin FLCs were one of the first tumor markers for multiple myeloma. Since their discovery, a great deal of progress has been made in identifying new protein markers for detecting, monitoring, and determining prognosis for multiple myeloma. Ways to detect these new markers include serum protein

electrophoresis and determination of serum FLC by nephelometry and other methods. Also important are assays for light chain/heavy chain-specific intact immunoglobulin subsets: IgGκ, IgGλ, IgAκ, and IgAλ (heavy/light chain immunoassays or HLC).

KEYWORDS: Electrophoresis, Immunofixation, Nephelometry, Immunoglobulins, multiple myeloma.

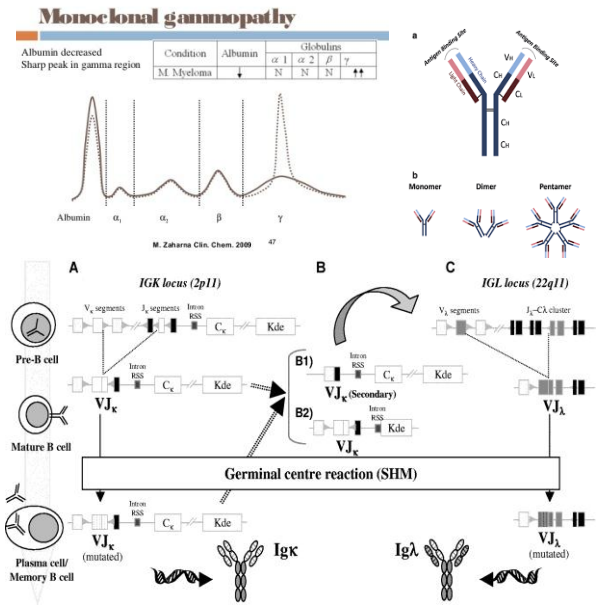
INTRODUCTION

All SPEP methods generally allow for quantization of monoclonal protein fractions. Total nephelometry immunoglobulin measurements can be used to monitor disease progression at high concentrations; however, at lower monoclonal concentrations their utility is compromised by the presence of polyclonal immunoglobulins. Immunofixation electrophoresis can be roughly described as a qualitative method for visualizing classes of monoclonal immunoglobulins and is generally more sensitive than SPEP. In this method, serum immunoglobulins are separated by electrophoresis, and different lanes are exposed to antibodies against immunoglobulin classes which bind and fix the immunoglobulin bands for staining and visualization. Overall, IgG is the most prevalent heavy chain associated with multiple myeloma clones (52%), followed by IgA (21%). Approximately 20% of myeloma clones express FLC only. The less common IgD monoclonal protein heavy chain is observed in 2% of myeloma clones and IgM heavy is found in 0.5% of myeloma clones. Notably, approximately 2% of myelomas are biclonal and 2% do not exhibit appreciable monoclonal protein by SPEP or IFE and are considered non-secretory. IgE monoclonal proteins are extremely rare. Observation of FLC in IFE such as that shown in Figure 2 must be checked using antiserum against IgD and IgE to ensure that there is not an unobserved associated heavy chain present in the initial IFE electrophoresis analysis. The technique of immunosubtraction or selective removal of immunoglobulin subtypes by antibody capture and subsequent comparison of SPEP patterns has been utilized successfully to type monoclonal protein but may be less sensitive than IFE. Both SPEP and IFE analysis of monoclonal proteins have notable limitations. IFE requires experienced analysis and often exhibits immunoglobulin restrictions that mimic monoclonal protein or broad nonspecific areas of immunoglobulin migration and impede identification of monoclonal protein. Without corroborating biochemical or clinical evidence, care must be taken not to overcall as definitive monoclonal protein observed bands of moderate to weak intensity. There are also significant challenges with determining monoclonal protein concentrations by SPEP[1]. Among these are overlapping non-immunoglobulin proteins in serum protein electrophoresis, particularly in the case of monoclonal proteins, which run more anodal. Another limitation is that overlap of monoclonal proteins can make quantification by electrophoresis challenging and subjective. IgG clones typically migrate in the gamma region, giving rise to the Gaussian gamma region pattern seen in normal SPEP analysis. However, IgA and IgM monoclonal proteins often run more anodal than IgG, overlapping other serum proteins (nonspecific IgA clones give rise to the beta bridging pattern in cirrhosis). The presence of non-tumor polyclonal immunoglobulins (Ig'κ and Ig'λ) of a given isotype potentially obscures clinical assessment of low concentrations of monoclonal protein. SPEP determinations can exhibit relatively high imprecision at concentrations below 10.0 g/L. Care must be taken not to introduce plasma into protein electrophoresis, as the presence of fibrinogen can give a false band in the beta gamma region which can be reversed upon addition of thrombin or ethanol. Additionally, the presence of non-tumor polyclonal immunoglobulins potentially obscures clinical assessment of low concentrations of monoclonal protein, and albumin determinations may be significantly higher relative to chemical dye binding methods in the presence of substantial monoclonal protein. Although monoclonal serum FLC can be quantified by SPEP in some cases, the use of a serum FLC nephelometric or turbidimetric polyclonal immunoassay has become standard practice for measuring free kappa (κ) and free lambda (λ) light chains in monoclonal gammopathies and other patient specimens. Alternative monoclonal FLC assays are also becoming available. FLC assays are designed to be specific to free immunoglobulin light chains and not react with light chains bound to heavy chains. In addition to high sensitivity and specificity, another advantage of serum FLC assays is that they can be used to calculate κ/λ ratios[2]. Furthermore, the short half life of FLCs (2–6 hours) and relative in vitro stability (several weeks in refrigerated serum) also are useful analytical characteristics. While current serum FLC assays are automated and have well-established reference intervals, they also have some limitations. These include potential non-linear dilution of monoclonal FLCs in the assay, and the possibility of antigen excess of the nephelometric assay. Antigen excess can lead to potentially disastrous and misleading analytical reduction of measured FLC concentrations even down to relatively normal concentrations when high concentrations actually exist. In cases of possible antigen excess where there is potential for falsely low results, such as on a new patient—especially in the concurrent presence of substantial FLC by IFE, or high concentrations of associated total heavy chain—further dilution of the specimen should be considered.

MATERIAL AND METHODS

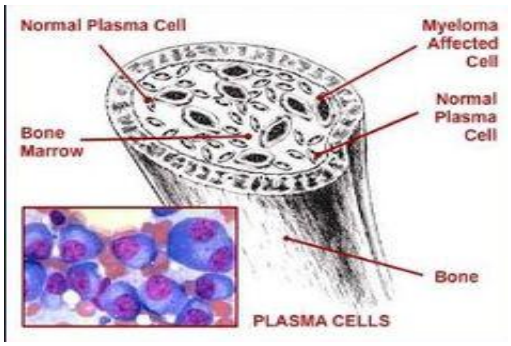
Twenty clinically diagnosed multiple myeloma patients attending the OPD or admitted indoor of GGS Medical College and Hospital, Faridkot were put under serum Protein Electrophoresis Pattern after getting their due permission. Capillary electrophoresis was utilized to find out their existence. Microalbuminuria was found out in the urine by Nyco card reader. Serum samples were stored at -20°C until thawed for the current study;

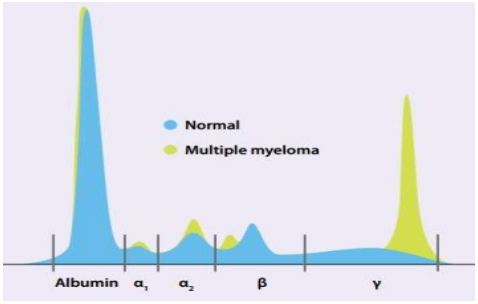
previous work has demonstrated the stability of FLC concentrations in serum samples over many years[21] and apparent stability in serum samples[12] Serum protein electrophoresis (SPE) and Immunofixation electrophoresis (IFE) were undertaken using the capillary electrophoresis .Serum κ and λ FLC concentrations were measured by nephelometry, all sera were assessed with SPE and FLC immunoassays; samples with abnormal results were investigated further by IFE. Urine of patients with suspected multiple myeloma was assessed for monoclonal FLCs by Immunofixation. All of these patients had abnormal serum FLC ratios[3]. The modified FLC ratio range increased the specificity of the assays (from 93% to 99%). Receiver operating characteristic (ROC) curve analysis was used to examine the sensitivity and specificity of utilizing the standard reference range for the FLC ratio versus the proposed reference range.



OBSERVATION

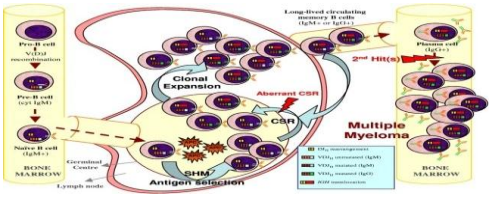
Since introduction of the first commercially available serum FLC assay, the clinical value of these tests has been well demonstrated. Most myeloma symptomatic patients (95%) exhibit abnormal κ/λ FLC ratios (FLCr). The value of serum FLC in detecting and screening for disease has been consistently verified, such that FLC measurements and ratios were included in the IMWG diagnostic guidelines. Additionally, serum FLC determination is used commonly to monitor disease progression during treatment, although there is no consensus on how FLC determinations should best be utilized in monitoring protocols. In addition to their role in diagnosing multiple myeloma and in monitoring disease progression and treatment, serum FLCr also provide information on prognosis for multiple myeloma patients at several points in disease survival. Evidence shows serum FLCr and absolute FLC of the involved light chain to be significantly prognostic for patient survival.[4] These ratios have also been shown to be independent and can be combined with risk stratification from the international staging system. Other studies have found serum FLCr prognostic in the rate and degree of normalization following treatment. Normalization of serum FLCr has also been included in the definition of stringent complete response in the IMWG response criteria. Abnormal serum FLCr along with increased clonal bone plasma marrow cells and elevated serum monoclonal protein have also been associated with increased risk of disease progression in smoldering multiple myeloma. Like smoldering myeloma, serum FLC has been incorporated into myeloma working group guidelines as a risk stratification factor. In addition, serum FLC measurements have shown utility in determining the prognosis of and in detecting and monitoring light chain amyloidosis. As such they quantitatively measure concentrations of specific antibody species such as IgGκ, IgGλ, IgAκ, and IgAλ. Of note, the Heavylite assay is not specific to one monoclonal protein. However, the presence of monoclonal protein is usually indicated by an abnormal heavy light chain kappa/lambda ratio.[5] There is substantial indication that heavy light chain Ig'κ/Ig'λ ratios may have diagnostic and monitoring value for multiple myeloma. They also may be valuable in monitoring disease progression, but there are no consensus guidelines on their use.[9] These heavy light chain assays appear to correlate well with total immunoglobulin measurements. In concordance with IFE, monoclonal immunoglobulins are typically quantified by SPEP and total immunoglobulin determinations, although universal correlation between these methods is not achieved. SPEP presents an analytical challenge in that the presence of overlapping non-immunoglobulin proteins makes it difficult to accurately determine monoclonal protein concentration. Myeloma patients also experience changes in hematocrit and blood volume, resulting in variable serum concentrations of immunoglobulins independent of tumor production, as well as variable of IgG brought on by differing clearance rates at high monoclonal concentrations. Measuring heavy light chain (Ig'κ/Ig'λ) ratios may alleviate these challenges. Recent reports have also suggested that heavy light chain ratios and suppressed concentrations of non-clonal heavy light chain proteins of the same class are of apparent prognostic significance in MGUS and in multiple myeloma.[6]





DISCUSSION

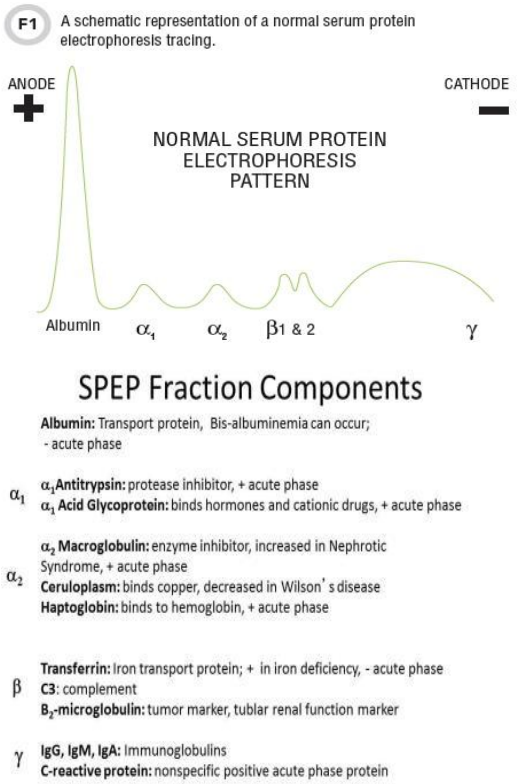
The purpose of this study was to assess serum free light chain immunoassays as an aid in the diagnosis of multiple myeloma in patients with severe renal failure. All 20 patients who were diagnosed with multiple myeloma were identified as abnormal using the assays. The assays indicate the presence of monoclonal FLCs by comparing the quantitative measurement of κ FLCs with λ FLCs, as a κ/λ FLC ratio. In patients with monoclonal κ FLC production the ratio is increased and in patients with monoclonal λ FLC the ratio is decreased. The sensitivity of the assays in this setting (100%) was not unexpected as previous studies have reported greater sensitivity for serum versus urine detection of monoclonal FLC in both myeloma and AL amyloidosis. Before serum FLC assays became available, urine analysis was the preferred method for identifying monoclonal FLC production in routine hematological screens for multiple myeloma and other lymphoproliferative disorders. However, the collection of urine samples, particularly 24 hour collections, is frequently problematic. In one screening study, urine samples were lacking for more than half the population] and in our study population urine samples were only provided for 24 of 41 myeloma patients. In an analysis of 428 patients with monoclonal FLCs in their urine, Katzmann *et al* found that the combination of serum electrophoresis tests and serum FLC analysis identified all patients requiring treatment and could, therefore, remove the requirement for urine analysis when screening [7]. Notably, the urine from one of the myeloma patients in our study was reported as normal despite a clearly abnormal serum FLC concentration. This study, therefore, provides further evidence that serum FLC assays may have greater utility for identifying monoclonal FLC production than urine analysis. The utility of a screening assay in practice however, is a function of its specificity as well as its sensitivity. In patients with renal failure, as glomerular filtration reduces, renal clearance of all FLCs will decrease. This results in longer serum half-lives and an increase in the κ/λ FLC ratio. Previous work we have undertaken demonstrated that in patients with renal failure, with no evidence of monoclonal proteins, the median FLC ratio was increased to 1.1 (range 0.37–3.1) from that of the published control population of 0.58 (0.26–1.65)[17]. We hypothesized that extending the reference range for the FLC ratio, to take into account this influence of renal function, would increase the specificity of the assay in patients with dialysis-dependent renal failure. Use of the published reference range for the FLC ratio, 0.26–1.65, gave the assay a specificity of 93% for patients with myeloma. This improved to 99% with the proposed extended reference range (0.37–3.1), indicating the new range may have a practical benefit by reducing the number of false positives. Interpretation of FLC ratios between 1.65 and 3.0 is currently difficult. We would propose checking the patient's renal function[2]. If normal, a ratio in this range may be indicative of a monoclonal process and further laboratory and clinical investigation will be appropriate.[8] If the renal function is abnormal, a ratio in the range of 1.65–3.0 is probably a consequence of the renal impairment; however further investigation of some patients may be appropriate, particularly if AL-amyloidosis is suspected. An interesting observation of this study was that the patients with cast nephropathy had higher absolute levels of the monoclonal free light chain type than the myeloma patients with other renal pathologies. Although this difference did not reach significance it adds further evidence to the findings of Bergner *et al* who demonstrated that urinary FLC concentrations are higher in patients with cast nephropathy compared with other FLC related renal pathologies. As with the findings of previous studies, cast nephropathy was the predominant cause of dialysis-dependent renal failure in the patients with multiple myeloma who had biopsies reported historically, myeloma patients with biopsy proven cast nephropathy have less than 25% chance of renal recovery and a significantly worse overall survival. Early reversal of the renal failure however, improves patient survival Novel therapies, currently under evaluation; aim to increase these renal recovery rates and patient survival. The focus of the new treatments is to rapidly reduce serum FLC concentrations, by either effective chemotherapy alone or in combination with direct removal of FLCs by high-cut-off hemodialysis.[9] Success however, is likely to depend upon early diagnosis and intervention; as animal models have indicated that within one month of obstruction, by a cast, irreversible damage has occurred to the nephron. The role of serum FLCs in management of patients with multiple myeloma and renal failure may expand beyond that of a diagnostic tool and management guide to that of an ndependent indicator of prognosis as eloquently demonstrated by Kyrtsonis et al in the general myeloma population.



RESULTS

Measurement of serum FLC concentrations and calculation of the serum κ/λ ratio is a convenient, sensitive and specific method for identifying monoclonal FLC production in patients with multiple myeloma and

acute renal failure. Rapid diagnosis in these patients will allow early initiation of disease specific treatment, such as chemotherapy plus or minus therapies for direct removal of FLCs. Immunoglobulin free light chains (FLCs) are by-products of immunoglobulin synthesis and in normal subjects are released into the circulation in small quantities.[1] The FLCs are then rapidly removed by renal clearance.[3] In patients with multiple myeloma, however, the clonal proliferation of plasma cells can produce FLCs in quantities thousands of times higher than normal[3] These monoclonal FLCs often result in renal pathologies, most importantly cast nephropathy. Indeed, multiple myeloma is the hematological malignancy most commonly associated with acute kidney injury (AKI). It has been proposed that the combination of multiple myeloma and AKI should be treated as a medical emergency with prompt diagnosis and intervention to avoid irreversible renal failure.[8]. However, the standard screening tests for myeloma, serum protein electrophoresis (SPE) and urine Bence Jones protein analysis are not always requested or reported promptly. Recently, immunoassays which measure the concentration of FLCs in serum have been incorporated into hematological screening algorithms for myeloma.[9–11]. These FLC assays are automated and allow same-day analysis and reporting of results. With these assays, the presence of monoclonal FLC production is indicated when the ratio of kappa (κ) to lambda (λ) serum FLCs is outside the reference range of 0.26–1.65.[2] The presence of an abnormal FLC ratio, suggestive of monoclonal FLCs production can occur in the settings of both intact immunoglobulin multiple myeloma and light chain only multiple myeloma. The identification of monoclonal protein production is not proof of multiple myeloma, but indicates that further investigations are required (principally a bone marrow biopsy and skeletal survey). For patients presenting with AKI, more rapid identification of multiple myeloma may lead to earlier interventions and improved patient outcome. However, there are no reported evaluations of the diagnostic utility of FLC assays in this setting.[3] One complicating factor is that patients with renal impairment can have κ/λ FLC ratios slightly above the reference range with no other evidence of monoclonal proteins.[4] This reflects a change in the dynamics of serum FLC clearance in renal failure. In normal subjects, the clearance of FLC from the serum is dominated by renal removal of FLCs which is preferential to the smaller, monomer, κ molecules. This gives a shorter serum half-life for κ and a median κ/λ FLC ratio of approximately 0.6. As the kidneys fail, however, the non-preferential reticulo-endothial route forms an increasing proportion of the FLC clearance .This results in a more similar serum half-life for the two FLCs and the FLC ratio therefore becomes increasingly influenced by the underlying production rates, by the plasma cells.[2] There are approximately twice as many κ producing cells as there are λ cells and this results in a ratio of total κ to total λ in the serum of approximately 1.8.[5]As expected, FLC analysis of sera from 688 patients with pre-dialysis, chronic kidney disease but no evidence of monoclonal immunoglobulin production (by serum Immunofixation electrophoresis) demonstrated the serum κ and λ FLC concentrations increased with decreasing renal function, FLC ranges: 3–251 mg/L and 1–251 mg/L, respectively[2]. The κ/λ FLC ratio increased with each increasing chronic kidney disease stage, through stages: 1–5 (population's serum creatinine: 56–875 μmol/L; estimated GFR: 6–128 mL/min/1.73 m^2). The median κ/λ FLC ratio of the population was 1.1 with a 100% range of 0.37–3.1. [11] This change in the ratio could reduce the diagnostic utility of FLC analysis in renal impairment.[5] We propose that modifying the κ/λ reference range to 0.37–3.1 may improve the diagnostic specificity when investigating patients with renal failure. The aim of this study was to evaluate serum FLC measurement as a diagnostic tool for detecting monoclonal FLCs and underlying multiple myeloma, in patients with dialysis-dependent AKI. The sensitivity and specificity of the published reference range was compared with the proposed renal failure reference range.



CONCLUSION

The measurement of serum FLCs can be a practical and highly sensitive aid in the identification of myeloma as the underlying pathology in patients with severe renal failure. Using an extended renal failure reference range for the FLC ratio increased the specificity of the assays. The diagnostic accuracy of these assays and their rapid laboratory turn-around time should aid nephrologists in their assessment of acute renal failure. The techniques used for serum and urine protein electrophoresis have improved significantly in both detection and resolution

during the past 70 years. The more sophisticated techniques of isoelectric focusing, Immunofixation and quantification of immunoglobulins are important in a work-up of a patient suspected of myeloma. Assay of serum free light chains provides an additional tool which can assist the laboratory in this process.

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