Review

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Laboratory testing requirements for diagnosis and follow-up of multiple myeloma and related plasma cell dyscrasias

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Abstract: Monoclonal immunoglobulins are markers of plasma cell proliferative diseases and have been described as the first (and perhaps best) serological tumor marker. The unique structure of each monoclonal protein makes them highly specific for each plasma cell clone. The difficulties of using monoclonal proteins for diagnosing and monitoring multiple myeloma, however, stem from the diverse disease presentations and broad range of serum protein concentrations and molecular weights. Because of these challenges, no single test can confidently diagnose or monitor all patients. Panels of tests have been recommended for sensitivity and efficiency. In this review we discuss the various disease presentations and the use of various tests such as protein electrophoresis and immunofixation electrophoresis as well as immunoglobulin quantitation, free light chain quantitation, and heavy-light chain quantitation by immuno-nephelometry. The choice of tests for inclusion in diagnostic and monitoring panels may need to be tailored to each patient, and examples are provided. The panel currently recommended for diagnostic screening is serum protein electrophoresis, immunofixation electrophoresis, and free light chain quantitation.

Keywords: free light chain assays; heavy/light chain assays; immunofixation; M-protein follow-up; M-spike; multiple myeloma; protein electrophoresis.

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Monoclonal gammopathies overview and categorization

Immunoglobulins are produced by plasma cells, and clonal plasma cell proliferative diseases usually secrete a monoclonal immunoglobulin (M-protein) that can be used as a serologic "tumor" marker. Because of this secreted monoclonal immunoglobulin, these diseases are also called monoclonal gammopathies. The secreted proteins can be used as a diagnostic tool for the identification of the clone of plasma cells as well as a quantitative marker to follow the course of the disease and response to therapy. Unlike most serologic tumor markers, M-proteins are extremely diverse. The M-proteins each have unique variable region sequences and the molecules may range from pentameric IgM (~900,000 Daltons) to monomeric free light chains (~24,000 Daltons). In addition, although some plasma cell proliferative disorders present with M-protein concentrations of grams per liter of serum, others have little or virtually no circulating M-protein. It is this diversity of structure and concentration that make this clonal marker so interesting and challenging.

Plasma cell proliferative diseases have different presentations, treatments, and outcomes. Table 1 lists by decreasing frequency of diagnosis 39,929 monoclonal gammopathies that were detected in the Mayo Clinic practice between 1960 and 2008 [1]. These disorders are often categorized as protein/low-tumor-burden diseases, premalignant disorders, and malignancies [2].

The low-tumor-burden diseases represent a group of monoclonal gammopathies in which there may not be a large clonal proliferation of plasma cells but in which the cell products cause pathology. This concept has been described for the first time in 2006 [3] and applies to conditions such as immunoglobulin light chain amyloidosis (AL). Laboratory testing requirements for those will be discussed in a separate chapter in this *CCLM* special issue.

Table 1: Prevalence of monoclonal gammopathies in the Mayo Clinic practice.

Monoclonal gammopathies: Mayo Clinic 1960–2008					
Disease	**Number of cases**	**%**	**Classification**		
			Malignant	**Protein/low tumor burden**	**Premalignant**
MGUS	23,179	58.0			X
Multiple myeloma	6974	17.5	X		
Primary amyloidosis	3781	9.5		X	
SMM	1494	3.7			X
Lymphoproliferative disease	1298	3.3	X		
Macroglobulinemia	940	2.4	X		
Plasmacytoma	774	1.9	X		
Bence Jones proteinuria	450	1.1			X
Cryoglobulinemia	379	0.9		X	
POEMS syndrome	217	0.5		X	
Light chain deposition disease	113	0.3		X	
Plasma cell leukemia	90	0.2	X		
Cold agglutinin disease	74	0.2		X	
Acquired Fanconi syndrome	43	0.1		X	
Scleromyxedema	31	0.1		X	
BHPW	31	0.1		X	
Heavy-chain disease	31	0.1	X		
Capillary leak syndrome	29	0.1		X	
Total	39,929	100			

MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; POEMS, polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes; BHPW, benign hypergammaglobulinemia purpura of Waldenström's. Adapted from reference [1].

Pre-malignant disorders include monoclonal gammopathies of undetermined significance (MGUS) and smoldering multiple myeloma (SMM). Although these conditions do not manifest symptoms that can be attributed to the plasma cell clone or monoclonal protein, they are called "pre-malignant" disorders because virtually all cases of multiple myeloma (MM) are now considered to have been preceded by MGUS or SMM as many as 8 or more years earlier [4–6]. Although there is significant overlap, the distinctions of MGUS, SMM, and MM are made because the diseases have different biology. MGUS is usually detected as an incidental finding when protein electrophoresis is done as part of laboratory studies for a wide range of symptoms [7]. It is a common disorder with a prevalence of 3%–4% in the Caucasian population over the age of 50 [8], occurs more frequently in men than women, and is at least 2-fold more prevalent in Africans and African Americans compared to Caucasians [9]. By definition the monoclonal protein is <30 g/L (3 g/dL) and bone marrow (if performed) contains <10% monoclonal plasma cells. In the Mayo Clinic practice MGUS represents 58% of all newly diagnosed monoclonal gammopathies (Table 1) [1].

Smoldering multiple myeloma is thought to be an intermediate stage between MGUS and multiple myeloma having >10% bone marrow plasma cells and/or >30 g/L monoclonal serum protein but no myeloma symptoms. The risk of progression to multiple myeloma is larger than for MGUS, at about 10% per year in the first 5 years of diagnosis [10], 3% in the next 5 years and 1% per year for the next 15 years. It may represent almost 14% of all patients with myeloma [11]. Diagnosis requires the absence of end-organ damage attributable to the clonal plasma cell disorder, and is considered a heterogeneous condition composed of patients with multiple myeloma that have not yet manifested clinical symptoms as well as patients with MGUS-like stable disease.

Both MGUS and SMM can progress to the malignant disease multiple myeloma. Low risk factors for progression of MGUS into MM are small M-spikes (<15 g/L), γ heavy chain, and normal free light chains (FLC) κ to λ ratio. MGUS with subtle abnormalities such as fuzzy gamma (γ) bands in serum protein electrophoresis (SPE) have been termed immunofixation electrophoresis (IFE) MGUS. It appears that IFE MGUS has a comparable risk to MGUS with small M-spikes [12]. In the Mayo Clinic study of southeastern Minnesota 42% of MGUS patients were low risk for all three factors and these low risk MGUS patients had a 0.1% per year risk instead of a 1% per year risk of progression [13].

Multiple myeloma (MM) is the most common malignant plasma cell disease and is second to non-Hodgkin lymphoma as the most common hematologic malignancy [14]. In the US population it is estimated that 23,000 new cases of MM will be diagnosed in 2015. MM is a disease of older people with the median age at diagnosis being 69 years and is rare in people younger than 40 [15, 16]. As in MGUS, the incidence is slightly higher in men and twice as high in Africans and African Americans than in Caucasians [17]. The racial disparities as well as family studies suggest genetic factors, but environmental factors such as exposure to agricultural and petroleum industry chemicals have also been shown to be risk factors [18]. Myeloma cells reside in the bone marrow, often suppress other marrow elements, and may cause destructive bone lesions. The presence of a large amount of bone marrow plasma cells (>10%) is not in itself sufficient for the diagnosis of MM. Until recently the distinction between MM and SMM required symptoms due to the plasma cell clone. In a series of 1027 sequential patients, the symptoms at diagnosis were anemia (73%), bone pain (58%), elevated creatinine (48%), fatigue/weakness (32%), hypercalcemia (28%), and/or weight loss (24%) [16]. The challenges of diagnosing multiple myeloma include the need for clinicopathological symptoms; summarized by the acronym CRAB: increased serum Calcium concentration, Renal insufficiency, Anemia, and lytic Bone lesions. In multiple myeloma, early detection is associated with 15% better overall survival [19] and less severe complications [20]. In order to intervene in high-risk SMM prior to end organ damage, the International Myeloma Working Group (IMWG) has recently updated the definition of multiple myeloma by adding three biomarker-characterized myeloma defining events to the CRAB acronym: 1) presence of bone marrow plasma cells >60% as a differentiation from smoldering multiple myeloma; 2) serum of involved FLC ratio is >100; 3) magnetic resonance imaging (MRI) with more than one skeletal focal lesion (Table 2). The updated criteria are justified by their prediction of early progression and as improved treatment options have become available in the last decade [21].

The other malignant diseases listed in Table 1 include lymphoproliferative disease, plasmacytoma, plasma cell

Table 2: Summary of the updated criteria for end-organ damage by the International Multiple Myeloma Working Group for the diagnosis of multiple myeloma.

Definition		Measurement recommendation
Myeloma defining events characterized by CRAB		
C	Hypercalcemia	Serum calcium >0.25 mmol/L (1 mg/dL) higher than the upper limit of the reference interval (RI, 2.15–2.55 mmol/L or 8.6–10.2 mg/dL, using photometric method)
R	Renal damage	Use of measured or estimated glomerular filtration rate (GFR) calculated with MDRD or CKD-EPI equations <40 mL/min instead of using a fixed serum creatinine concentration of 173 μmol/L (2 mg/dL)
A	Anemia	Hemoglobin value >20 g/L (2 g/dL) below the lower limit of the age/gender reference interval, or an absolute hemoglobin value below 100 g/L (10 g/dL)
B	Bone lesions	Osteoporosis with compression fractures attributable to a clonal plasma cell disorder or clear evidence of one or more osteolytic bone lesions (≥5 mm in size). IMWG recommends that PET-CT, low-dose whole body CT, 18F-fuorodeoxyglucose (FDG)-PET or MRI of the whole body or spine be done in all patients. Imaging modality should be determined after availability and resources. Caution should be taken to avoid over interpretation of equivocal lesions; additional studies suggested in 3–6 months before therapy is initiated
One or more of the following biomarkers of malignancy (BOM)		
1	>60% plasma cells on bone marrow	Measured by conventional bone marrow aspirate or biopsy; do not use flow cytometry. When a discrepancy exists between biopsy and aspirate, use the higher value
2	Involved free light chain ratio >100	Nephelometry measurement of κ and λ light chains. Minimum concentration of involved light chain (κ or λ) should be ≥100 mg/L (10 mg/dL)
3	More than one skeletal focal lesion	Magnetic resonance imaging (MRI) preferred, additional studies with CT or PET-CT should be considered if lesions are small (<5 mm)

IMWG, International Myeloma Working Group; MRI, magnetic resonance imaging; CT, X-ray computed tomography; PET-CT, positron emission tomography-computed tomography; FDG, 18F-fluorodeoxyglucose; MDRD, Modification in Diet in Renal Disease Study; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration. Adapted from reference [21].

leukemia, and the heavy chain diseases. A small percentage of B-cell lymphomas and leukemias will produce a monoclonal immunoglobulin usually found in low concentrations in serum. Plasmacytomas are solitary lesions that usually present with pain at the site of the lesion which contains clonal plasma cells in the absence of any diffuse bone marrow involvement or CRAB symptoms. Plasmacytomas often have no or small amounts of secreted monoclonal protein and usually respond well to radiation. Within 2 years, however, approximately 50% will recur as multiple myeloma [22]. Plasma cell leukemia is a rare and aggressive form of myeloma with high numbers of circulating plasma cells. Interestingly, approximately 35% of plasma cell leukemia patients will have light chain only and 10% will be non-secretory [23]. Heavy chain diseases are exceedingly rare and are identified by the presence of a monoclonal heavy chain with no corresponding monoclonal light chain on IFE [24]. The heavy chain is often detected in significant concentrations in the urine, and urinary IFE is a useful confirmatory assay. Many heavy chain disease patients have lost the ability to synthesize light chains, but some patients have mutations in the heavy chain that results in the inability to bind light chain. These latter patients may therefore present with an isolated monoclonal heavy chain detected by IFE plus monoclonal free light chain as detected by a significantly abnormal FLC κ to λ ratio [25]. In a series of γ-heavy chain disease patients, the heavy/light chain assays resulted in poor recovery using IgG κ and IgG λ specific reagents, indicating that a significant amount of IgG was not quantitated. The inability to account for all of the IgG immunoglobulin by IgG κ and IgG λ reagents is a red flag to search for free γ heavy chain [26].

The definitions of MGUS, SMM, and MM, the distributions of monoclonal immunoglobulins as well as the risk of progression are summarized in Table 3. Free light chains and monoclonal IgD occur in MM at about 4-fold the frequency as in MGUS. The laboratory detection of monoclonal free light chains or monoclonal IgD should always raise the suspicion of serious disease. Laboratorians who see samples that are mostly from MGUS patients and few samples from MM patients are almost always surprised by the relatively high frequency of light chain multiple myeloma (LCMM) and non-secretory multiple myeloma (NSMM) among MM patients. Another clear difference is in the frequency of IgM MGUS and IgM MM. IgM multiple myelomas are rare. IgM MGUS documented only by IFE (no quantifiable M-spike) may be persistent in 73% of patients, however, no progression was documented for a group of 87 patients during a median follow-up of 2.6 years (range 0.1–13 years) [12]. Some IgM MGUS patients present with neuropathies. Most IgM disease that progresses to malignancy is classified as Waldenström's macroglobulinemia (WM). WM is a disease of clonal lymphoplasmacytic cells and is a distinct clinical-pathologic entity from MM. WM may present with indications of serum hyperviscosity, and commonly develops symptoms related to infiltration of hematopoietic tissues as anemia, lymphadenopathy and hepatosplenomegaly.

Another group of patients who suffer from motor or sensory peripheral neuropathies may have small amounts of M-proteins in the serum that require immunofixation for detection. Some of these patients have POEMS syndrome (also called Crow-Fukase syndrome), an acronym for peripheral neuropathy, organomegaly, endocrine dysfunction along with an M-protein and skin hyperpigmentation. Unlike screening for MM or WM, cases of POEMS syndrome and individuals with peripheral neuropathies who have myelin-associated glycoprotein (MAG) monoclonal antibodies require that IFE be performed on serum for detection [27].

Laboratory diagnosis of monoclonal gammopathies

The diversity of plasma cell proliferative diseases, their secreted proteins, and the concentrations of M-proteins make this a challenging tumor marker. No single assay can effectively diagnose and monitor plasma cell proliferative diseases, and the laboratory needs to define strategies that encompass the spectrum of disease presentations [2]. Traditionally, the detection of M-proteins relies on the ability to differentiate between monoclonal and polyclonal immunoglobulins using agarose gel or capillary zone electrophoretic assays. In addition, IFE in agarose and immunosubtraction electrophoresis (ISE) by capillary zone electrophoresis are used to characterize the immunoglobulin heavy and/or light chain isotypes. Nephelometric methods can aid in the detection of M-proteins and can complement some of the weaknesses of electrophoretic techniques.

Serum protein electrophoresis (SPE)

In normal serum the protein electrophoretic γ fraction has a broad, Gaussian distribution due to the thousands of clones of plasma cells that secrete immunoglobulin into serum. Quantitation of the γ fraction provides information about hypo- or hypergammaglobulinemia. In monoclonal gammopathies, the M-protein is visualized as a restricted area of migration in the electrophoresis pattern. Although

Table 3: Definition of monoclonal gammopathies and diagnostic tests.

	Definition	Immunoglobulin isotypes associated with disease	Risk of progression
Monoclonal gammopathy of undetermined significance (MGUS)	Monoclonal protein is <30 g/L (3 g/dL) and bone marrow (if performed) contains <10% monoclonal plasma cells. There is no end-organ damage defined by CRAB or BOM nor AL amyloidosis. Three types of MGUS have been defined: – Non-IgM MGUS – IgM MGUS – Light-chain MGUS	IgG, 59% IgA, 12% IgM, 18% IgD, 0.5% Biclonal, 5% Light chain, 6%	MGUS has a 1% per year risk of progression, and the risk of progression remains at 1% per year regardless of length of follow-up Non-IgM MGUS have an overall risk of progression of 1% per year of progressing to SMM, MM or AL IgM MGUS has a 1.5% risk of progressing mainly to WM but also to lymphoma or AL amyloidosis Light Chain MGUS has a 0.3% risk of progressing to Light Chain MM and AL amyloidosis
Smoldering multiple myeloma (SMM)	No CRAB end-organ damage symptoms nor BOM. SMM is distinguished from MGUS by having an M-spike (IgG or IgA) >30 g/L (3 g/dL) or urinary M-protein >500 mg/24 h and/or monoclonal bone marrow plasma cells between 10% and 60%		SMM patients progress at a rate of 10% per year in the first 5 years of follow-up, and the remaining SMM progress at approximately 1%–2% per year
Multiple myeloma (MM)	Intact immunoglobulin or light chain MM are defined by abnormal plasma cells in the bone marrow ≥10% or biopsy proven bony or extramedullary plasmacytoma and end-organ damage defined by CRAB or BOM or amyloidosis. Non-secretory MM is defined by absence of serum or urinary M-protein and have no detectable abnormalities on serum or urine immunofixation. Approximately 70% of patients have an abnormal FLC ratio	IgG, 52% IgA, 21% IgM, 0.5% IgD, 2% Biclonal, 2% Light chain, 20% Non-secretory, 3%	About 80% of multiple myeloma originates from non-IgM MGUS, and 20% from light chain MGUS
Waldenström's Macroglobulinemia	Defined as a distinct clinicopathological entity demonstrating lymphoplasmacytic lymphoma in the bone marrow with the presence of a monoclonal IgM protein regardless of the serum concentration. Ten percent or more of the bone marrow biopsy demonstrate infiltration with plasma cell differentiation (lymphoplasmacytic features or lymphoplasmacytic lymphoma)	IgM, 100% 80% are κ light chain	Prevalence is rare in African-Americans and 25% of patients are asymptomatic at diagnosis. Five year overall survival is of approximately 60%

CRAB, hypercalcemia, renal damage, anemia and bone lesions; BOM, biomarkers of malignancy; FLC, free light chains.

a discrete band on SPE is rarely a false-positive result, all patients with a localized band or non-homogeneous distribution on SPE require IFE or some other method to confirm and characterize the heavy and light chains. In a cohort of sequential 1027 patients diagnosed with multiple myeloma, the M-spike mobility was distributed in the following order: 54% of M-spikes were in the γ fraction, 12% in β-γ junction, 13% in β and 1% in the α-2 fraction [16]. A monoclonal protein may be present even when the total protein, the β and γ fractions, as well as the quantitation of immunoglobulins by nephelometry are all within the reference intervals, and that was the case in 11% of

the study cohort for newly diagnosed MM [16]. Challenges for SPE include patients with LCMM, who represented 18%–20% of all patients with MM [16]. They present with numerous clonal plasma cells secreting monoclonal light chain, but the serum concentration is often low due to the low molecular weight of the free light chain that is rapidly cleared from circulation by the kidneys. Other challenging disease presentations for SPE include monoclonal proteins that migrate in the β fraction of the protein electrophoresis, such as 30% of all IgAs that may hide under the β peak, or the rarer IgD myeloma cases, where the M-protein appears very small or not evident at all.

Immunofixation electrophoresis (IFE)

For most patients with a discrete M-spike in protein electrophoresis, IFE is straightforward. In current practice, IFE is performed with antisera to IgG, IgA, IgM, total κ and total λ. If a monoclonal light chain is visualized without a corresponding heavy chain, an additional immunofixation gel is performed with antisera to delta and epsilon heavy chains. IFE has improved sensitivity [limit of detection of approximately 100 mg/L (10 mg/dL)] when compared to SPE [limit of detection of 1000 mg/L (100 mg/dL)], at approximately 10-fold, and therefore a negative SPE result does not entirely rule out a monoclonal gammopathy and should be followed by IFE.

Immunoglobulin quantitation

Although the nephelometric quantitation of immunoglobulins is a simple automated method, it measures both monoclonal and polyclonal immunoglobulins. The diagnostic use of nephelometry for identification of monoclonal proteins is not recommended because it will include monoclonal and polyclonal immunoglobulins and is of no value for biclonal and triclonal gammopathies. There is, however, a role for nephelometry for detecting and monitoring free light chains and for monitoring large IgG M-spikes (>30 g/L or 3 g/dL) when saturation of the dye binding poses as a limitation. Quantitation of IgA by nephelometry is usually similar to the M-spike obtained by protein electrophoresis. The IgM concentration, on the other hand, may be up to 2-fold greater than expected from the M-protein measured in SPE [28, 29]. Quantitation of immunoglobulins is more useful than SPE for detection of hypogammaglobulinemia, and the best approaches to use nephelometry or SPE are described in Table 4. The use of mono-specific antisera to bound and free (total) κ and λ light chains will only detect excess light chains of very large concentrations of intact monoclonal proteins, and, moreover, there is a significant variation regarding the potency of the antisera from different sources [30, 31]. The use of assays to quantitate total κ and/or λ light chains is not recommended. In practice total light chains are used only in urine specimens as a quality control tool for monitoring urine M-spikes. The measurement of total light chains by nephelometry should not be confused with the quantitation of free light chains.

Free light chains (FLC) or Bence Jones protein quantitation

Light chains have a molecular weight of approximately 24 kDa and contain 210–220 amino acids comprising a variable and a constant domain. Free κ chains (e.g. not bound to heavy chain) often circulate as monomers, but they may exist as dimers that are linked non-covalently or a mixture of monomers and dimers. Free λ chains, on the other hand, commonly circulate as covalently linked dimers. Light chains are normally produced by plasma cells in molar excess to heavy chains. In normal individuals approximately 500 mg of FLC are synthesized each

Table 4: Uses and limitations of immunoglobulin quantitation by nephelometry and serum protein electrophoresis M-spike to monitor monoclonal gammopathies.

Immunoglobulin quantitation by nephelometry	Serum protein electrophoresis M-spike
Can be used when there is little polyclonal Ig synthesis	Small abnormalities will include considerable amount of polyclonal background
Should be used when IgA M-protein migrates in the beta fraction	M-spike usually equivalent to nephelometry for IgA
Should be used when IgG M-spike is >30 g/L	M-spike may saturate gel staining if IgG >30 g/L
Note that IgM is usually 1.8 times higher than protein electrophoresis IgM M-spike	Imprecision is high when M-spike <10 g/L
	Best way to monitor biclonal and triclonal gammopathies

day from bone marrow and lymph node cells [32, 33]. The excess amount of FLC is rapidly secreted and then cleared by the kidneys. The FLC have a shorter serum half-life (2–6 h) [34, 35] than intact immunoglobulins (IgG – 23 days; IgA – 5.8 days; IgM – 5.1 days; IgD – 2.8 days; IgE – 2.3 days) [36]. Quantitative FLC assays use antisera directed against epitopes that are exposed only when the light chains are free (unbound to heavy chain) in solution. These "cryptic" sites are involved in the very tight, non-covalent binding of light chains to heavy chains. The antisera have approximately a 10,000-fold stronger binding avidity for FLC compared to light chains contained within intact immunoglobulin molecules [37]. That means that FLC immunoassays can be used to specifically quantitate FLC even in the presence of large concentrations of polyclonal serum immunoglobulins. The approach to diagnosis is to quantitate both κ FLC and λ FLC concentrations and use the ratio of κ to λ FLC concentrations to detect unbalanced light chain synthesis. Although the κ to λ synthesis rate is approximately 1.8:1, because of more rapid clearance of κ FLC, the median FLC κ/λ ratio is 0.9 (0.26–1.65). This approach to detect unbalanced FLC serum concentrations has proven surprisingly sensitive for detecting clonal free light chain diseases. Abnormal serum FLC ratios have been detected in 100% of patients with LCMM, 80%–95% of AL patients, and 60%–70% of patients with non-secretory multiple myeloma [38–41]. In spite of the sensitivity of FLC assays in free light chain diseases, abnormal serum FLC ratios have only been detected in 90%–95% of intact immunoglobulin MM and 40% of MGUS [39]. Since these two patient groups have easily detectable serum M-proteins by SPE and IFE, it is clear that not all monoclonal gammopathies secrete excess FLC and that a combination of tests is be needed to benefit from each test's strengths.

Urine assays

Dipstick tests are the most commonly used screen for urine protein. However, the indicator dye tetrabromophenol binds to albumin and therefore these assays are often insensitive to monoclonal proteins excreted in the urine. Random urine collections may be used as an initial screen due to being easier to collect, however, a confirmatory 24-h urine study is useful to document baseline renal function and the total amount of protein excreted per day. A total protein measurement (pyrogallol red method) is not sufficient to document monoclonal protein in urine, as a nephrotic pattern of proteinuria (≥3 g/24 h) seen in amyloidosis or light chain deposition disease cannot be differentiated from the myeloma pattern, which is the predominance of the M-protein, and commonly urine protein electrophoresis and immunofixation are needed.

In IgG MM, >75% of patients have monoclonal FLC detected by urine IFE (Bence Jones proteinuria) [42]. Patients with LCMM may present with low concentrations of M-protein in circulation. For such cases, it has been important to test both serum and urine. A 24-h urine collection may allow the visualization and quantitation of the M-protein that has been rapidly cleared by the kidneys. The use of serum FLC assays, however, has reduced the need for urine electrophoretic assays for detection and quantitation of monoclonal free light chains. The International Myeloma Working Group now suggests using serum FLC as part of the diagnostic criteria instead of urine protein electrophoresis and IFE if MM is suspected [21]. If urine studies are done for monitoring patients, IFE must be done to confirm clonality due to the increased potential of false-positive electrophoresis bands in urine compared to serum. In addition, monoclonal proteins in urine may present as wider bands in electrophoresis than in serum, usually due to degradation and longer application time in the gel. Occasionally there may be two discrete light chain bands, which may be a monoclonal light chain plus a monoclonal immunoglobulin or fragment from serum, or monomers and dimers of the same monoclonal light chain in the absence of a corresponding heavy chain [36]. Also, if heavy chain disease is suspected from serum IFE, urine IFE is a useful confirmatory test. Measurement of urine total light chains by nephelometry is less sensitive than urine IFE, and is rarely ordered as a diagnostic test for urinary monoclonal protein.

Serum viscosity

Serum viscometry is recommended when there is more than 40 g/L of IgM monoclonal protein, more than 50 g/L of IgA, or more than 60 g/L IgG protein and in any patient with oronasal bleeding, blurred vision, or neurological symptoms suggestive of a hyperviscosity syndrome. Symptoms of hyperviscosity are rare unless the value is more than 5 centipoises (cp), but some patients with a value of 10 cp or more do not have symptoms [43]. The signs of the hyperviscosity syndrome include segmental dilation of retinal veins and flame-shaped hemorrhages, oronasal bleeding, blurring or loss of vision, headaches, vertigo, nystagmus, decreased hearing, ataxia, paresthesias, diplopia, somnolence, stupor, or coma [44]. Waldenström's macroglobulinemia is the most common cause of hyperviscosity and accounts for more than 80% of cases. Multiple myeloma

of IgA type or, rarely, IgG may produce hyperviscosity [44, 45]. The Ostwald-11 viscometer is a viable instrument for viscometry measurement, but a Wells-Brookfield viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA) or Sonoclot Coagulation Analyzer (Sienco Inc., Wheat Ridge, CO, USA) are preferred because they are more accurate and require less serum (approx. 1 mL). In addition, determinations can be made much more rapidly, especially if the viscosity of the serum is high.

Cryoglobulins

Cryoglobulins are proteins that precipitate when cooled and dissolve when heated. Testing for cryoglobulins has remained the same over the last several decades. If a precipitate or gel is seen in the serum tube after 24 h incubation in an ice bath in a cold room, the tube is centrifuged and the cryocrit concentration is determined. The precipitate is washed and IFE is performed on the resuspended, warmed cryoprecipitate. Cryoglobulins are classified as follows: type I (monoclonal: IgG, IgM, IgA, or rarely monoclonal light chains); type II (mixed: two or more immunoglobulins, of which one is monoclonal); and type III (polyclonal: in which no monoclonal protein is found) [46]. In most cases, monoclonal cryoglobulins are IgM or IgG, but IgA and Bence Jones cryoglobulins have been reported. Unexpectedly, many patients with large amounts of cryoglobulin are completely asymptomatic, whereas others with small monoclonal cryoglobulins in the range of 10–20 g/L (1–2 g/dL) have pain, purpura, Raynaud's phenomenon, cyanosis, and even ulceration and sloughing of skin and subcutaneous tissues on exposure to the cold [47, 48]. The temperature at which the cryoglobulin precipitates is much more important than the amount of protein. Most commonly, mixed cryoglobulins (type II) consist of monoclonal IgM with rheumatoid factor activity and polyclonal IgG, but IgG-IgG and IgA-IgG combinations have been reported [49]. Patients with mixed cryoglobulinemia frequently have vasculitis, glomerulonephritis, or lymphoproliferative or chronic infectious processes, like hepatitis B or hepatitis C infections [50, 51].

Screening panels for M-proteins

There have been a number of studies identifying the best approach for detection of monoclonal proteins [2, 52–56]. The Mayo Clinic group performed a large retrospective study in which patients were selected with an assortment of plasma cell proliferative diseases and who also had SPE, IFE, and FLC as well as urine protein electrophoresis and IFE performed at the time of diagnosis. The cohort consisted of 1851 patients with various monoclonal gammopathies [MM, WM, SMM, MGUS, plasmacytoma, POEMS syndrome, Immunoglobulin light chain primary amyloidosis (AL), and light chain deposition disease (LCDD)] [2]. The data illustrated in Table 5 allows a determination of which patients would have had M-proteins detected by the various tests singly or in combination. The three columns of SPE, IFE, or FLC as single serum tests detected 79%, 87%, and 74% of the patients. A panel with all three serum assays plus urine IFE detects 98.6% of the cases. Interestingly, if urine assays are removed from the diagnostic panel, there is no decrease in sensitivity for

Table 5: Diagnostic sensitivities of tests or combination of tests used for screening of monoclonal gammopathies.

Diagnosis	Number of subjects, n	Single assays			Combination of assays		
		SPE, %	Serum IFE, %	Serum FLC, %	SPE+FLC, %	SPE+IFE +FLC; no urine, %	All serum and urine tests, %
All	1877	79.0	87.0	74.3	94.3	97.4	98.6
MM	467	87.6	94.4	98.6	100	100	100
WM	26	100	100	73.1	100	100	100
SMM	191	94.2	98.4	81.2	99.5	100	100
MGUS	524	81.9	92.8	42.4	88.7	97.1	100
Plasmacytoma	29	72.4	72.4	55.2	86.2	89.7	89.7
POEMS	31	74.2	96.8	9.7	74.2	96.8	96.8
AL	581	65.9	73.8	88.3	96.2	97.1	98.1
LCDD	18	55.6	55.6	77.8	77.8	77.8	83.3

SPE, serum protein electrophoresis; IFE, immunofixation electrophoresis; FLC, free light chains by nephelometry; MM, multiple myeloma; WM, Waldenström's macroglobulinemia; SMM, smoldering multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; POEMS, polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes; AL, immunoglobulin light chain primary amyloidosis; LCDD, light chain deposition disease. Adapted from reference [1].

patients with MM, macroglobulinemia, plasmacytoma, POEMS, or SMM. This and other studies have led the International Myeloma Working Group to recommend a screening panel of SPE, IFE, and FLC: the inclusion of diagnostic urine testing is only recommended if AL is suspected. In addition, once an M-protein has been detected, analysis of urine may be required [57]. This diagnostic panel of 3 serum assays simplifies sample acquisition for the patient and reduces costs for the laboratory since only a single sample is transported and accessioned, and no pre-analytic centrifugation and concentration of urine is needed. A simplified diagnostic panel of SPE and FLC is probably the simplest screening panel that should be considered until more sensitive detection systems are developed for the clinical laboratory. The use of mass spectrometry has been recently suggested by the application of matrix assisted laser desorption ionization – time of flight instruments (MALDI-TOF) as a rapid and inexpensive method for detecting monoclonal proteins [58].

Monitoring monoclonal gammopathies

Once an M-protein has been detected and a specific diagnosis has been determined, the quantitation of the M-protein can be used as a marker of the plasma cell clone's response to therapy, stability, or progression. Just as there are multiple tests that may be required for diagnosing monoclonal gammopathies there are multiple assays for measuring M-proteins; and just as in diagnostic testing, the smaller the M-protein the harder it is to quantify the abnormality. Quantitative tests include SPE for M-spike measurement and immunonephelometry for Ig quantitation, FLC concentration, and heavy/light chain (HLC) measurements.

If a serum M-spike and/or urine M-spike is present in large enough concentration, it can be easily quantitated by electrophoresis. The advantage of using the SPE M-spike when the abnormality is in the γ fraction is that the fractionation of the M-spike separates the M-protein from most of the polyclonal background. The International Myeloma Working Group defines serum M-spikes >10 g/L and urine M spikes >200 mg/24 h as being "measurable". "Measureable" means that the size of the M-spike is large enough to be reliably quantified and is also large enough to document a decline in the M-protein and therefore, a hematological response. The variability of the quantitation of the SPE M-spike increases substantially when the M-spike values are <10 g/L [59].

There are also disease presentations in which there is suppression of polyclonal immunoglobulin synthesis. In cases where there is very little polyclonal Ig, the quantitation of immunoglobulin by immunoassay (IgG, IgA, or IgM) can also be used to monitor hematologic disease. Serum M-spike and immunoglobulin quantitation are not, however, always equivalent and clinicians should not go back and forth between methods. Many clinicians order both SPE and Ig quantitation as a quality check to see that both change in similar ways [28].

Not all patients can be monitored by SPE M-spikes and/or quantitative immunoglobulins. Some patients have small concentrations of M-protein. In these cases quantitation of the M-protein will include substantial amounts of polyclonal immunoglobulins. The M-spike will be more enriched for the monoclonal protein than quantitative Ig, but the M-protein will still be a minority of the M-spike. As this M-spike gets smaller in response to therapy the laboratory may still recognize the SPE abnormality but has to make a judgment when to stop fractionating the M-spike. When the monoclonal protein appears to be <1/4 to 1/3 of the M-spike quantitation, it is usual practice to stop fractionating an M-spike and only report a "small monoclonal protein that we are unable to quantitate". That is not a quantitative result, but quantitation could be misleading as the polyclonal background changes. Newer methods such as modeling M-spikes with tangent gating, HLC quantitation, and quantitation by mass spectrometry may solve quantitation of smaller concentrations of monoclonal proteins.

When monoclonal IgA or IgM proteins migrate in the β fraction instead of the γ fraction, monitoring becomes even more complex. If migration is in the β fraction, the protein may not be visualized because it is obscured by transferrin, C3, and other serum proteins. For IgA monoclonal gammopathies, when the IgA band is indistinguishable from the normal β components, a common approach is to fractionate the β band as an M-spike if the β fraction is >20 g/L (2 g/dL). As the amount of monoclonal protein changes over time, the arbitrary gating of the M-spike is confusing for clinicians and patients. It is useful in these cases to use quantitative immunoglobulins to assist monitoring of IgA and IgM proteins. The use of capillary electrophoresis and immunosubtraction give good resolution of the β-1 and β-2 peaks, and may be able to isolate the abnormality or estimate the portion of the β region that has been subtracted and provide a reliable value [60]. Changes in the size of the β fraction and the quantitative Ig or the immunosubtraction electrophoresis supported measurement should be concordant. We recommend that clinicians order both immunofixation and electrophoresis to document the

continued presence of the monoclonal IgA as well as nephelometric quantitation of IgA. The complementary use of nephelometry for quantitation is supported in the 2014 International Myeloma Working Group recommendations [61]. When the IgA concentration by nephelometry falls into the reference range it is not clear if monoclonal IgA is still present and therefore SPE plus IFE is needed or heavy-light chain (HLC) assays can be used to quantitate as well as document the monoclonal protein.

New HLC reagents that can separately quantitate IgG κ and IgG λ, IgA κ and IgA λ, and IgM κ and IgM λ may be useful for monitoring monoclonal proteins migrating in the β fraction. The heavy-light, isotype-specific κ to λ ratio has been proposed as a potential monitoring method for IgA or IgM M-proteins migrating in the β fraction [62, 63]. Even though the monoclonal protein is not visualized by SPE, an abnormal heavy-light κ to λ ratio (rHLC) indicates excess clonal synthesis analogous to the use of the FLC κ to λ ratio [64–66]. Although the assay is not sensitive enough to use as a routine screening method for MM, a 97% sensitivity observed in IgA MM and IgA MGUS indicates that almost all IgA MM patients can be monitored by HLC for both clonality and quantitation using the IgA HLC assay [63]. If the rHLC is abnormal, no immunofixation is needed and the involved heavy/light chain (iHLC) provides quantitation. In a recent study with 30 diagnostic and post-treatment sera from β-migrating IgA MM cases, the correlation of IgA iHLC was better with total IgA quantitation (r=0.97) than with SPE M-spike (r=0.87). Considering that approximately 21% of MM patients have an IgA monoclonal protein [16], and that in the study cohort, 50% of all samples had a normal IgA quantitation, IgA HLC assays have a potential role for monitoring myeloma in this population [63].

The international guidelines for monitoring monoclonal gammopathies suggest that if the serum M-spike is <10 g/L and the urine M-spike is <200 mg/24 h, then the FLC assays should be considered for monitoring [57]. The guidelines for FLC quantitation state that in order to monitor a "measurable" free light chain to document response to treatment, the FLC ratio should be abnormal and the concentration of the monoclonal FLC should be >100 mg/L (10 mg/dL).

Definitions of partial, complete, and stringent responses when treating MM are also addressed by guidelines [67]. A complete response is defined as a normal serum and urine IFE (as well as bone marrow plasma cells under 5%). A stringent complete response is a complete response plus a normal FLC κ to λ ratio and a negative bone marrow aspirate measured by immunofluorescence or immunohistochemistry. Currently, partial responses are defined by the following decreases in measurable M-proteins: 50% decrease in serum M-spike, 90% decrease in urine M-spike, and/or 50% decrease in serum FLC. In order to test these partial response guidelines, the variability of long-term, sequential serum and urine samples in MM patients who have already reached stable partial remissions [59] was studied and results indicated that the biologic and disease-related variation in these stable patients was 8% for serum M-spikes, 12% for immunoglobulin quantitation, 28% for serum monoclonal FLC concentrations, and 36% for urine M-spikes. This variability data suggests that the serum FLC quantitation has variability that behaves more like the urine M-spike. The concentration of serum FLC and urine M-spike both depend on the synthesis of FLC and then processing of the low molecular weight light chain by the kidney. In contrast, the serum M-spike depends on the synthesis and very slow clearance of intact Ig. The serum M-spike measurement has the lowest biological variability, and when possible, it is the parameter of choice for monitoring disease.

Once the monoclonal protein concentration falls below measurable amounts and is too low to be quantitated as an M-spike or abnormal FLC or HLC, then monitoring becomes the qualitative detection (yes/no) of minimal residual disease. The recent measurement of very small concentrations of intact monoclonal protein using monoclonal immunoglobulin Rapid Accurate Mass Measurement (miRAMM) by time-of-flight mass spectrometry has suggested that the limits for serum detection of minimal residual disease in MM may be lowered [68–70]. Because mass spectrometry is quantitative, these lower detection limits may increase our ability to monitor small concentrations of monoclonal proteins. The use of mass spectrometry for detection of the intact immunoglobulin light chain is more sensitive than sCR (e.g. SPE, IFE and FLC are all normal) and its resolution of exact mass allows quantitation of the monoclonal protein separate from polyclonal background [70]. Because the detected light chain is part of the intact immunoglobulin, the biological variability of this method may actually be similar to serum M-spike quantitation rather than serum FLC.

Final remarks

Although plasma cell dyscrasias are diverse, the suggested panel of SPE, IFE, and FLC provides relatively simple and sensitive diagnostic testing. However, a few suggestions are

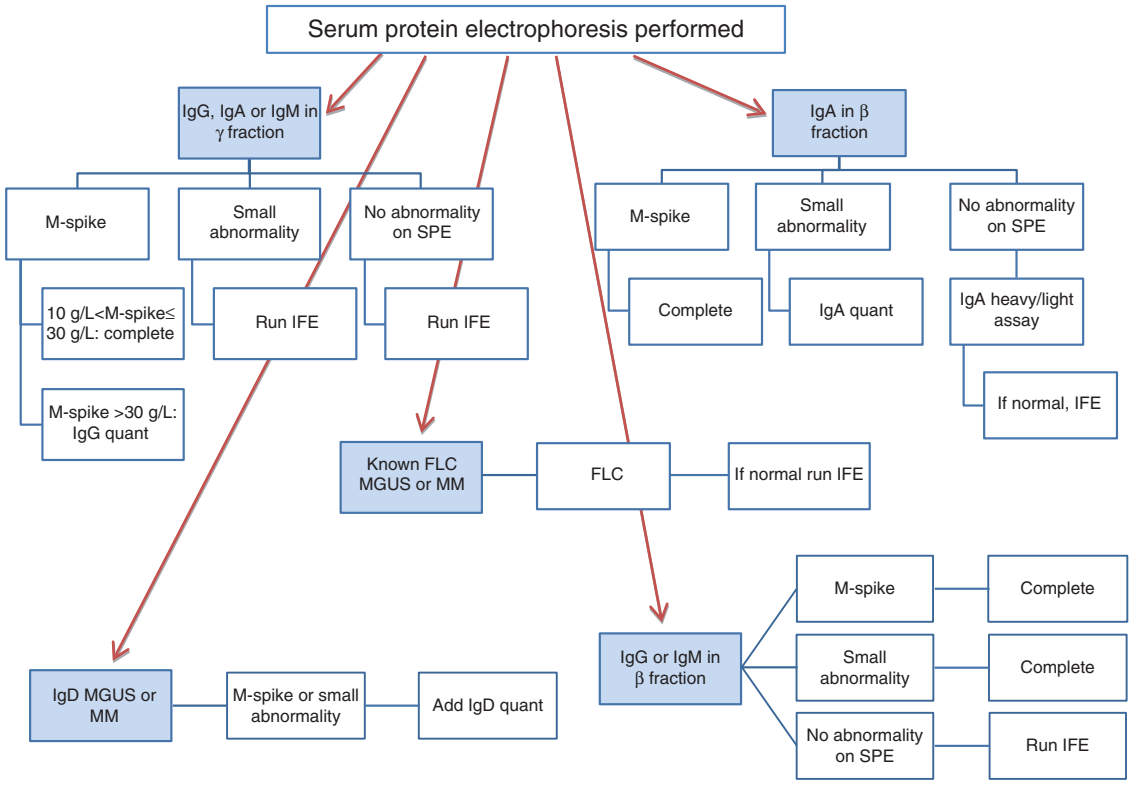

Figure 1: Suggested testing algorithm for monitoring monoclonal gammopathies with current assays.
A small abnormality is defined as a visual abnormality on the SPE pattern, unable to be quantitated. SPE, serum protein electrophoresis; IFE, immunofixation electrophoresis; quant, immunoglobulin quantitation by nephelometry; FLC, free light chains quantitated by nephelometry.

provided here that could encourage a more logical pattern of ordering diagnostic tests and/or monitoring methods. Order formats can provide several embedded clinical situations allowing clinicians to request testing that is specific to their patient, such as "rule out monoclonal protein". Monitoring panels for monoclonal gammopathies should all start with a protein electrophoresis and further testing can be reflexed after an M-spike, small abnormality or no abnormality are identified. If the reflex decisions can be made by the laboratory, the history retrieval and diagnostic isotype will be necessary in evaluating what tests are needed for monitoring a specific monoclonal gammopathy (Figure 1). New mass spectrometry methods may eventually be used side-by-side to conventional methods fully validated and translated into routine use.

In summary, as an update in current testing requirements for monoclonal gammopathies, we have described the use of SPE, IFE, and nephelometric methods for Ig, FLC, and HLC for detection and monitoring plasma cell dyscrasias. The use of SPE and FLC as an initial screening will diagnose 100% of myeloma cases, and seems to be a cost effective and viable option for the majority of laboratories worldwide.

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