New Tools for Diagnosis and Monitoring of Multiple Myeloma

Jesús F. San-Miguel, MD, PhD, Bruno Paiva, PhD, and Norma C. Gutiérrez, MD, PhD

OVERVIEW

This article reviews the most relevant techniques currently used for the evaluation of patients with multiple myeloma. Although bone marrow morphologic examination and electrophoretic analysis of the monoclonal paraprotein and conventional x-rays remain the “gold standard” techniques for fast, accurate, and cost-effective diagnosis, other assays such as molecular cytogenetics, immunophenotyping, MRI, and PET-CT may contribute to a better assessment of patients with myeloma. Here, we will discuss not only the contribution of each technique to differential diagnosis of monoclonal gammopathies, but also the value of each parameter for determining prognosis and for monitoring treatment efficacy. In addition, possible technical pitfalls inherent to each technique will be analyzed.

Diagnosis of multiple myeloma (MM) requires the examination of bone marrow showing plasma cell infiltration, detection, and quantification of monoclonal protein in the serum or urine and evidence of end-organ damage (i.e., hypercalcemia, renal insufficiency, anemia, or bone lesions).1,2 Diagnostic assays have three main objectives: to contribute to the diagnosis and differential diagnosis of monoclonal gammopathies, to yield information about prognostic factors in order to facilitate the therapeutic decision-making process, and to provide appropriate tools to monitor treatment efficacy. It should be noted that many of the laboratory parameters contribute to more than one objective. In this review we have grouped the different diagnostic and prognostic assays into five areas: protein analysis, morphology, immunophenotyping, genetics and cytogenetics, and imaging techniques (i.e., MRI, PET/CT). An overview of diagnostic tools is provided in Table 1.

PROTEIN ANALYSIS

Measurement of the serum and urine monoclonal immunoglobulin (MC) has been a mainstay in the treatment of patients with multiple myeloma (MM). Agarose gel electrophoresis or capillary zone electrophoresis of serum and urine is preferred to screen for the presence of MC, but quantitation of serum immunoglobulins (particularly those uninvolved) by nephelometry should also be performed.1 Bence-Jones or immunoglobulin D (IgD) myeloma should be suspected in the absence of serum MC but severe hypogammaglobulinemia.3 Once the MC is detected, the heavy and light chain isotypes must be identified by immunofixation. If negative for IgG, IgA, and IgM but positive for kappa or lambda, an immunofixation for IgD or IgE should be performed.3 Twenty-four–hour urine collection is mandatory to assess the total amount of protein excreted. An aliquot must be concentrated (150 to 200 fold) for electrophoresis (detection of monoclonal light chain) and immunofixation (isotype identification). The lowest detected level of M-protein by electrophoresis ranges between 0.2 g/L and 0.6 g/L, whereas for serum immunofixation it ranges between 0.12 g/L and 0.25 g/L. According to the International Myeloma Working Group,2 a serum MC of 3 g/dL or greater distinguishes patients with smoldering myeloma from patients with monoclonal gammopathy of undetermined significance (MGUS), but it is not a requisite to define symptomatic disease. The size of the MC does not have prognostic influence in MM.4 During the past decade the measurement of serum immunoglobulin-free light chains (FLC) has become part of routine clinical testing. An abnormal serum FLC ratio indicates the presence of clonality in approximately one-third of patients with MGUS and in 90% or greater of those patients with smoldering and symptomatic myeloma (including light chain disease).5 The assay is particularly indicated for the treatment (diagnosis and follow-up) of patients with nonsecretory and oligosecretory myeloma, as well as amyloidosis, but measurement of serum FLC levels does not obviate the need for 24-hour urine studies.1,5 Note-worthy, a highly abnormal serum FLC ratio predicts for shorter time to progression in patients with MGUS and smoldering myeloma, as well as for inferior survival rates for patients with active myeloma.5

The variation in the size of the MC by electrophoresis is the major criterion to define response to treatment in myeloma. Complete response (CR) criteria requires (among others) negative immunofixation of serum and urine, and stringent
CR requires the CR criteria plus a normal serum FLC ratio. Achievement of CR is now considered one of the strongest prognostic markers in myeloma, both in the transplant and nontransplant settings. The stringent CR criteria has failed to unequivocally demonstrate a superior prognostic value compared with conventional CR, which might be partially explained by the presence of false-positive results driven by oligoclonal expansions after therapy. This particular drawback could be overcome with a novel assay that separately identifies the different light chain types of each immunoglobulin class (that is, IgG-kappa, IgG-lambda, IgA-kappa, and IgA-lambda), therefore capable of quantitate involved and uninvolved immunoglobulins (e.g., IgG-lambda, IgA-kappa, and IgA-lambda for a patient with IgG-kappa disease). Recent studies have shown that the new heavy/light chain assay affords additional prognostic information in MGUS and active myeloma; however, it should be noted that the major contribution for prognostication comes precisely from the quantitation of the uninvolved immunoglobulins, thereby highlighting the importance of initial polyclonal immunoglobulin suppression and later recovery to identify patients with MGUS who have higher risk of progression or increased likelihood of long-term survival in active myeloma, respectively.

### KEY POINTS

- Conventional morphology, protein electrophoresis, and skeletal survey remain the standard of care in the diagnosis and treatment of patients with myeloma, but novel cellular, serologic, and imaging assays have found their way into the clinic.
- Serum-free light chain and the new heavy/light chain assays are particularly valuable for diagnosis and follow-up of oligosecretory myelomas; however, these are not currently a substitute for the 24-hour urine assay.
- Fluorescence in situ hybridization (FISH) analysis on purified plasma cells is mandatory at baseline for patient risk stratification and should only be repeated at relapse/progression for those patients initially classified as genetic standard risk.
- Flow cytometry immunophenotyping and allele-specific oligonucleotide polymerase chain reaction have contributed to the evaluation of minimal residual disease (MRD), which translated into definition of high-quality responses (immunophenotypic and molecular remission) associated with longer survival and with the possibility of monitoring consolidation and maintenance therapies.
- Novel imaging techniques (e.g., MRI or PET/CT) have progressively been incorporated into routine practice and might become a new standard in the future, particularly for identification of occult bone disease in smoldering myeloma and to exclude extramedullary disease for definition of complete response outside of the bone marrow.

### TABLE 1. Most Relevant Techniques Currently Used for the Evaluation of Patients with Multiple Myeloma

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Abbreviations: CR, complete response; FISH, fluorescence in situ hybridization; MGUS, monoclonal gammopathy of undetermined significance; MRD, minimal residual disease.

The urine M-protein should also be monitored during follow-up, even in patients without urinary excretion of paraprotein at diagnosis, because there are cases with light chain escape as the only sign of relapse. Albumin quantitation is important for staging, and the most accurate assay is nephelometry because electrophoresis might overestimate albumin concentration if there is a high MC.

### MORPHOLOGY

The estimation of bone marrow plasma cell infiltration is a major criterion for the diagnosis and differential diagnosis of...
MM and other plasma cell dyscrasias (e.g., solitary plasmacytoma).\textsuperscript{1,2,9,10} Recent consensus from the International Myeloma Working Group support that a patient with suspected MM should undergo a unilateral bone marrow aspirate (May-Grünewald Giemsa stained smears) and/or biopsy, and the diagnosis is confirmed when 10% or greater of plasma cells are detected.\textsuperscript{1,2} The use of bone marrow biopsies is probably a more accurate method (through CD138 staining) for evaluation of plasma cell infiltration because cytomorphology is more vulnerable to the heterogeneous distribution of plasma cells in the bone marrow, and the percentage of plasma cells may substantially vary depending on site of sample aspiration.\textsuperscript{3} This phenomenon could also help to explain the inconsistency of plasma cell quantification as a prognostic factor in symptomatic patients. However, for the definition of CR, morphologic assessment is mandatory and requires the presence of less than 5% of plasma cells.\textsuperscript{5} Immunoperoxidase staining, immunofluorescence, or flow cytometry immunophenotyping of the bone marrow aspirate can be used to establish the clonality of plasma cells; however, this is not a standard procedure.

In contrast with other hematologic malignancies, little attention in MM is paid to the morphologic characteristics of plasma cells (e.g., mature, intermediate, immature, plasmablasts).

**IMMUNOPHENOTYPING**

The multiparameter nature of flow cytometry allows the detection of clonal plasma cells through their aberrant phenotypes rather than by light chain restriction. aberrant phenotypes include: typically underexpression of CD19, CD27, CD38, CD45, and/or CD81; overexpression of CD28 and/or CD56; and asynchronous expression of CD117.\textsuperscript{9} The degree of clonality assessed by immunophenotyping (i.e., the balance between malignant and residual normal plasma cells) has become particularly relevant to the identification of patients with MGUS or smoldering myeloma who are at different risk of progression into symptomatic disease—higher risk for patients in whom almost all plasma cells are clonal (greater than 95%).\textsuperscript{11} In addition, immunophenotyping identifies prognostic-associated antigenic profiles (e.g., CD19\textsuperscript{+}, CD28\textsuperscript{+}, CD81\textsuperscript{+}, or CD117\textsuperscript{−} being associated with inferior outcome) as well as patient-specific phenotypic profiles informative for minimal residual disease (MRD) monitoring.\textsuperscript{9}

The CR rates have substantially improved with up-front treatment with novel agents and high-dose therapy followed by autologous stem cell transplantation\textsuperscript{12}; overall survival rates have also substantially improved for patients with myeloma.\textsuperscript{13} However, only a minor fraction of patients actually achieves long-term disease control (more than 10 years of disease-free survival),\textsuperscript{6} which underlies the persistence of MRD undetectable by conventional serologic and cytomorphologic techniques. The need for bone marrow confirmation of CR has been discussed, but important data showing that up to 14% of patients with immunofixation-negative CR might have have 5% or greater of plasma cells in the marrow confirms the need for its evaluation.\textsuperscript{14} However, in contrast with acute leukemias, conventional morphologic methods are generally not able to distinguish normal from clonal plasma cells. Moreover, the value of immunohistochemistry or immunofluorescence is rather limited in this setting because of the recovery of normal plasma cells after therapy that precludes the use of low-sensitive techniques. The unique features of multiparameter flow cytometry immunophenotyping, applicable to 90% or greater of patients and with a sensitivity to detect one or more tumor cells out of 10,000 normal cells render this an attractive approach for patient follow-up. From a clinical point of view, achieving an immunophenotypic CR (no residual aberrant plasma cells with a sensitivity limit of \(10^{-6}\)) predict for extended survival in younger patients undergoing intensive therapy\textsuperscript{15,16} and elderly patients treated with novel agents.\textsuperscript{16,17} Moreover, similar to the paradigm in other hematologic malignancies (e.g., acute lymphoblastic leukemia, chronic myeloid leukemia, and acute promyelocytic leukemia), risk assessment combining baseline evaluation through (cyto)genetics and MRD monitoring following up-front treatment provides accurate patient stratification—identifying those at risk of showing unsustained CR.\textsuperscript{15} Pitfalls of conventional flow cytometry include its lack of standardization and the need for experienced personnel. The EuroFlow Consortium is trying to overcome these drawbacks; moreover, the evolution from four-color into multidimensional flow cytometry with eight or more colors is increasing the specificity and sensitivity of MRD assessments. Regarding molecular polymerase chain reaction techniques, these are well standardized and are suitable for MRD monitoring, with several small studies showing the clinical significance of achieving a molecular CR.\textsuperscript{18,20} However, highly sensitive molecular approaches (\(10^{-5}\) to \(10^{-6}\)) rely on the design of allele-specific oligonucleotides, which are time and labor consuming, relatively expensive, and require quality DNA not only in post-treatment samples but also at baseline. The estimated applicability of this approach is approximately 70% in myeloma.\textsuperscript{18} Some aspects of current molecular approaches have the potential to be overcome by novel high-throughput gene sequencing, which already showed impressive results in acute lymphoblastic leukemia. It should be noted that MRD negativity (either by immunophenotypic or molecular techniques), even with a sensitivity of \(10^{-5}\) or higher, does not necessarily mean complete tumor eradication, particularly in a disease typically characterized by patchy marrow infiltration and extramedullary involvement. Therefore, to have a positive MRD result is a negative prognostic factor, but a negative MRD is not always associated with a favorable outcome. Nonetheless, there is an increasing interest in MRD monitoring as a tool for risk-adapted treatment, particularly in the consolidation and maintenance settings.
GENETICS AND CYTOGENETICS

As in other hematologic malignancies, cytogenetics has become one of the most important prognostic factors for MM. The advent of high-throughput methodologies for genomic analysis has greatly increased the variety of available technologies for investigating genetic abnormalities. Thus, modern whole-genome techniques such as comparative genomic hybridization, mapping arrays based on single nucleotide polymorphisms, and gene expression profiling have been added to the techniques of classical karyotyping and molecular cytogenetics based on fluorescence approaches. Nowadays, cytogenetic evaluation is mandatory in all patients with newly diagnosed MM and should always include interphase fluorescence in situ hybridization (FISH) in purified plasma cells or in combination with immunofluorescent detection of light chain-restricted plasma cells (clq-FISH). Cytogenetic abnormalities in MM can be classified in two main groups: translocations involving immunoglobulin heavy-chain locus (IGH) and genomic imbalances. Patients can have one or more of these abnormalities, and in general, over time, there is accumulation of new cytogenetic abnormalities.

IGH translocations are detectable in approximately 40% of patients. There is a notable diversity of chromosomal partners involved in IGH translocations. The most recurrently involved loci are 11q13 (CCND1) in 15%, 4p16 (FGFR3/MMSET) in 15%, and 16q23 (MAF) in 5% of cases. Several groups have demonstrated that t(4;14) is associated with poor survival. Patients with t(4;14) treated with either conventional or intensive chemotherapy display shorter event-free survival and overall survival times, but recent studies show that it may be possible to almost completely overcome the poor prognostic effect of t(4;14) using bortezomib-based regimens. However, recent analysis suggest that patients with t(4;14) make up a heterogeneous group. Thus, the Intergroupe Francophone du Myélome (IFM) discriminated a subset of these patients (approximately 45%) with both low beta2-microglobulin and high hemoglobin diagnosis who are experiencing prolonged survival after tandem transplant, thus benefitting from high-dose therapy. This heterogeneity has also been reported by the Arkansas group using the 70 gene-expression model, which enables a clear separation of two groups of t(4;14) patients with different overall survival. Regarding t(14;16), controversial results (because of its low frequency) have been reported using autologous transplant: the IFM did not confirm the poor prognostic value of t(14;16) in patients receiving a tandem-autologous transplantation approach, whereas a recent study from the MRC Myeloma IX trial showed a shorter survival time among patients with t(14;16) who were treated with autologous transplant.

MM is characterized by the frequent occurrence of chromosomal imbalances including gains and losses that lead to the classification into hyperdiploid and nonhyperdiploid subgroups, the former having a better prognosis. Several studies have shown that 1q gains is selected as an important and independent poor prognostic factor, although there are also series that have failed to confirm this. Monosomy 13/13q deletions (present in approximately 50% of patients) have been associated with short survival in almost all large series of patients treated with both conventional and high-dose therapy. However, this adverse prognosis come from its close association with other high-risk genetic features such as t(4;14), which harbors monosomy 13 in 80% of patients with MM. In fact, the Rb deletion on its own is not a negative prognostic factor. Although deletion of 17p, which includes the p53 locus, is less frequent in MM (occurring in approximately 10% of patients), it remains a strong prognostic factor that has been associated with a negative effect on survival in different treatment contexts. Extramedullary disease, which is commonly related to more aggressive disease, has a higher prevalence of 17p deletion.

Most of the genetic studies show a close association among chromosome abnormalities in MM. Recently, it has been shown that the cosegregation of multiple adverse genetic lesions confers the worst clinical prognosis. On the contrary, a recent study showed that the presence of trisomies in patients with high-risk cytogenetic abnormalities can almost fully abrogate the adverse prognostic effect of the additional abnormalities on MM survival.

There is general consensus that the same genetic abnormalities characteristic of poor prognosis at diagnosis may suggest poor outcome if detected at the time of relapse. Therefore, although routine genetic analysis should be confined to diagnostic myeloma samples, it should be carried out at relapse in those cases initially classified as genetic standard-risk, with the goal of identifying high-risk genetic features associated with adverse prognosis not present at diagnosis time. Finally, because the sensitivity limit of FISH assay is $10^{-2}$, it is not recommended for MRD analysis.

IMAGING STUDIES

Bone disease is the hallmark of MM, with up to 90% of patients developing osteolytic lesions during the course of their disease. Imaging is therefore mandatory in patients’ initial work-up, and bone disease distinguishes active treatment-requiring myeloma. Conventional skeletal survey has been considered during the past 4 decades as the gold standard for baseline evaluation of bone disease. A total of 13 plain radiographs should be performed including anteroposterior and lateral of skull, spine (cervical, thoracic, and lumbar) femora and humeri, plus posteroanterior of chest (focus on ribs and scapula) and pelvis. Special attention should be paid to lesions at risk of impending fracture. Almost 80% of patients with symptomatic myeloma will have radiologic evidence of skeletal involvement, but the technique lacks specificity (discrimination of benign causes of osteopenia) and sensitivity, because lytic lesions are only evident if greater than 30% of bone substance has been lost.

MRI is the most sensitive noninvasive imaging technique for detection of bone involvement in the spine. It also provides relevant information on the extent and nature of soft tissue disease and on the pattern of marrow infiltration (i.e.,
normal, focal, variegated, or diffuse). MRI is mandatory in (1) presumed diagnosis of solitary plasmacytoma; (2) symptomatic patients for a detailed evaluation of a painful area of the skeleton to look for a soft-tissue mass arising from a bone lesion; (3) suspicion of cord compression; and (4) before kyphoplasty. An MRI is recommended for patients with smoldering myeloma because it can detect occult lesions, which predict for faster progression to symptomatic disease. It is also recommended for patients with nonsecretory myeloma (for diagnosis and follow-up) and for patients with a vertebral collapse in the context of osteoporosis. Osteoporosis with compression fracture requires extensive evaluation by MRI. If a malignant lesion is detected, then the patient has symptomatic disease and requires antmyeloma treatment. Noteworthy, bone fracture may be the sequel of osteoporosis (particularly in elderly white women), and other criteria such as anemia or renal impairment should be considered to diagnose symptomatic myeloma. Occasionally, an MRI-assisted CT-guided biopsy of the collapsed vertebra is needed to make the diagnosis. High numbers of focal lesions (more than seven) or a diffuse MRI pattern are considered as adverse prognostic factors in patients with symptomatic myeloma.

CT scanning is more sensitive and faster than conventional skeletal survey, but the radiation dose delivered can be up to 3 times higher. Whole-body low-dose CT has recently been investigated, and it may be an alternative for patients for whom MRI is contraindicated. Similarly, CT scan should also be considered when MRI is unavailable for assessment of the spine or to clarify the extent of soft tissues. It is also valuable to clarify lytic lesion in the ribs, sternum, and scapulae, as well as to assist planning of radiotherapy or surgery.

The use of PET/CT is not recommended outside of clinical trials, although it may be informative regarding patients with elevated lactate dehydrogenase, Bence Jones protein escape, and rapidly recurrent disease with no marrow involvement, as well as for patients with suspected extramedullary myeloma. Recent studies showed an independent prognostic value of baseline fluorodeoxyglucose-PET/CT evaluation and of fluorodeoxyglucose suppression before and after high-dose therapy. Accordingly PET/CT may be useful to evaluate MRD outside of the bone marrow and to predict long-term outcomes.

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