Is This the Time to Introduce Minimal Residual Disease in Multiple Myeloma Clinical Practice?

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Abstract

Increasing therapeutic options and prolonged survival in multiple myeloma have raised interest in the concept of depth of response and its importance to predict patients’ outcomes. Although the efficacy of current treatment approaches has greatly improved in the past decade, the definition of complete response (CR) remains unaltered and continues to use conventional serological and morphologic techniques. That notwithstanding, there is growing interest in minimal residual disease (MRD) monitoring, which has emerged in recent years as one of the most relevant prognostic factors in multiple myeloma. MRD can be assessed both inside (e.g., immunophenotypic and molecular techniques) and outside the bone marrow (e.g., PET/CT). Here, we focus on flow- and molecular-based assays by which different cooperative groups have demonstrated the efficacy of MRD assessment to predict outcomes even among patients in CR, and irrespectively of disease risk. Although further standardization is still required, the time has come to implement MRD monitoring in prospective clinical trials as a sensitive tool to evaluate treatment efficacy and for risk-adapted treatment, particularly in the consolidation and maintenance settings. Here, we present a comprehensive and critical review on the methodologic aspects, specific characteristics, and clinical significance of MRD monitoring by flow cytometry, PCR, and next-generation sequencing. Clin Cancer Res; 21(9): 1–8. ©2015 AACR.

Introduction

In the past few decades, important changes have been introduced in the treatment of multiple myeloma, resulting in unprecedented complete response (CR) rates and prolonged progression-free survival (PFS) and overall survival (OS; refs. 1–3). In 2006, the International Myeloma Working Group proposed a new CR definition and introduced the normalization of serum-free light chains (sFLC) and absence of bone marrow clonal plasma cells (PC) by immunohistochemistry/immunofluorescence as additional requirements to define stringent CR (4). Recently, Kapoor and colleagues (5) demonstrated the added prognostic value of the stringent over conventional CR criteria, even though other groups have failed to show additional value of sFLC assessment among CR patients (6, 7). However, the sensitivity of immunohistochemistry/immunofluorescence is rather low (10^{-7}) due to the recovery of normal PCs after therapy that normalize k/λ ratios (8). Thus, it becomes evident that current criteria used to assess response lag behind the extraordinary evolution in the treatment of multiple myeloma patients, and more sensitive techniques are needed to detect true minimal residual disease (MRD) for new CR definitions. This change would help to improve monitoring of treatment efficacy and to avoid overtreatment and undertreatment, particularly during consolidation and maintenance.

Current techniques to monitor MRD can be divided into those measuring extramedullary versus those detecting intramedullary disease. MRI and 18-fluoro-deoxyglucose PET/CT have emerged as promising tools to measure extramedullary MRD. In particular, PET/CT has been shown to be of prognostic value as soon as day 7 of induction therapy (9) and more importantly, among patients in conventional CR after high-dose therapy/autologous stem cell transplantation (HD/TASCT; ref. 10). For detection of intramedullary disease, both multiparameter flow cytometry (MFC) immunophenotyping and molecular assessment of immunoglobulin rearrangements are the pivotal techniques. The present review focuses on current state-of-the-art intramedullary MRD monitoring in multiple myeloma.

Methodological Characterization of MRD Techniques

Multiparameter flow cytometry (MFC)

Simultaneous assessment of CD38 and CD138 represents the best marker combination for specific identification of PC (11–14). In the event of anti-CD38 and/or anti-CD138 therapies, other antigens such as CD229, CD319, or CD54 could be alternative markers for PC identification. Phenotypically aberrant clonal PCs typically show (i) underexpression of CD19, CD27, CD38, and/or CD45; (ii) overexpression of CD56; (iii) asynchronous expression of CD117 (15–24). Accordingly, these antigens are consensually used for the clinical study of bone marrow PCs (25), and at least one (8-color) combination of such markers is highly recommended for multiple myeloma flow-MRD monitoring. No single parameter reliably distinguishes clonal from
normal PCs; however, a multiparameter approach that evaluates all markers used in a single tube can readily identify PC aberrant phenotypes, provided a sufficient number of cellular events are evaluated in the flow cytometer (26).

Flow-based MRD monitoring has benefited from polychromatic cytometers that allow simultaneous assessment of ≥8 markers at the single-cell level which, coupled with novel software, results in more accurate discrimination of clonal versus normal PCs (27). The principal component analysis (PCA) of unified data files allows automatic multidimensional separation of all cellular clusters present in a sample. PCA-based interpretation of flow cytometric data facilitates the development of normal and tumor reference libraries, automated separation of populations within a sample, and prospective detection and tracking of any aberrant cell population. Such a strategy is likely the method of choice for accurate and semiautomated flow-MRD monitoring.

Allele-specific oligonucleotide PCR (ASO-PCR)

PCR-based detection of multiple myeloma MRD relies on the identification of persistent tumor cells through the amplification of the immunoglobulin heavy-chain (IGHV) gene rearrangement (28). Consensus primers binding to the framework regions of the IGHV (which are less variable than the complementary determinant regions, CDR) were initially used for the identification of residual disease. This approach amplifies the monoclonal IGHV rearrangement but also those from normal B cells, and thus the sensitivity reached (10⁻⁵–10⁻⁶) was insufficient for MRD detection. Afterwards, specific primers (ASO) complementary to the most variable regions of the IGVH rearrangement (CDR or complementary determinant regions) were used. The limit of detection with this approach was optimal (10⁻⁴–10⁻⁶), but the results obtained were merely qualitative (29, 30). The subsequent step was the implementation of real-time quantitative PCR strategies that allow the exact quantification of tumor cells in real time. This method is based on the use of an ASO primer (usually complementary to the CDR3 region) together with a consensus primer (frequently complementary to the joining region) and a fluorescent probe to monitor the amplification.

Next-generation sequencing (NGS)

In recent years, sequencing technologies that quickly perform millions of reads of DNA fragments have emerged. This technology not only allows to distinguish normal from tumor DNA, but also to detect previously known tumor-specific sequences within normal DNA fragments (i.e., MRD monitoring). Current NGS methods include (i) pyrosequencing, based on the luminometric detection of the pyrophosphate released when individual nucleotides are added to DNA templates from an emulsion PCR; (ii) multiplex sequencing-by-synthesis technology, which relies on light signals emitted during the re-synthesis of small DNA fragments previously produced by bridge amplification; and (iii) ion semiconductor sequencing, which detects hydrogen ions liberated during DNA polymerization. Using these techniques, rearranged B-cell receptor (BCR) and T-cell receptor (TCR) genes can be deeply sequenced (31–35). These methods use a consensus PCR to amplify all possible BCR or TCR rearrangements which, at diagnosis, allow identifying and sequencing monoclonal rearrangements (35). After therapy, monoclonal rearrangements can be investigated among thousands of normal cells through several millions reads, providing high specificity and sensitivity for MRD detection of BCR and TCR genes.

Advantages and Disadvantages of MRD Techniques

Multiparameter flow cytometry

An increasingly higher number of flow cytometry laboratories use digital instruments that provide simultaneous assessment of more parameters per tube (≥8), as well as a superior event acquisition and analysis of a greater cell numbers than previously seen with 4-color instruments. The availability of digital cytometers coupled with novel sample preparation methods allows for fast and cost-effective measurement of millions of leukocytes. Consequently, although previous MFC studies defined MRD as the presence of a discrete population of clonal PCs equal or above the 0.01% limit of detection, nowadays such thresholds stands at least at 0.001% (10⁻⁴). Because clonal PCs are readily distinguished from normal PCs on the basis of their aberrant phenotypes, flow-MRD is applicable to virtually all patients and does not require patients’ diagnostic phenotypic profile. Furthermore, the flow-MRD assays incorporate a quality check of the whole sample cellularity (i.e., B-cell precursors, erythroblasts, etc.), which is critical to ensure sample quality since hemodiluted bone marrow aspirates can lead to false-negative results. Even though hemodilution can be assessed with a microscope—providing that the sample in which hemodilution is being assessed is exactly the same as that in which MRD will be assessed—the possibility for MFC to simultaneously analyze MRD and hemodilution automatically in the same sample is more accurate and particularly attractive. The requirement for extensive expertise in MFC analysis and the lack of a well-standardized flow-MRD method have been mentioned as important disadvantages of MFC immunophenotyping. However, automated identification and characterization of cell populations, coupled with reference databases in the context of full technical standardization, have now emerged as the probable solution for these problems. There is now the unmet need for different MFC groups to adopt single, standardized, and validated antibody panels, sample processing, and cell-analysis methods such as those being developed by the EuroFlow consortium for MFC to become a universal and fully standardized option for MRD assessment. Another potential limitation of MFC could be to ignore potential multiple myeloma cancer stem cells outside the PC compartment (i.e., memory B cells; ref. 36). However, recent investigations conducted with optimized molecular assessment of clonal variable, joining, and diversity (VDJ) sequences among FACS-sorted peripheral blood B-cell subsets revealed that such clonotypic cells are either absent or present below the detection limits (10⁻⁶; ref. 37).

Regarding the potential impact of genetic heterogeneity and clonal tiding after treatment on the feasibility of MFC to detect MRD, it should be highlighted that the multidimensional approach of current flow cytometry immunophenotyping not only allows detection of clonal heterogeneity in approximately 30% of newly diagnosed patients, but also makes it possible to monitor all the different phenotypic subclones throughout a patient’s treatment, thereby assessing potential (phenotypical) clonal selection upon therapy. Nevertheless, it should be noted that according to the experience of the Medical Research Council (MRC) and the Grupo Español de Mieloma (GEM) groups based on large patient cohorts, there are no major antigenic shifts for consensus markers (e.g., CD19, CD38, CD45, or CD56) used to monitor MRD (GEM; unpublished data). Accordingly, potential clonal evolution throughout the course of treatment does not influence the efficacy of MFC-based MRD assessment.
Allele-specific oligonucleotide PCR

PCR approaches for MRD detection do not require an immediate sample processing because they are unaffected by pre-analytical biases such as loss of viable cells over time (38, 39). Furthermore, the MRD target used is based on the uniqueness of clonal IGHV rearrangements which leads to a high sensitivity, adequate for MRD detection ($10^{-5}$ to $10^{-8}$; refs. 40, 41). PCR strategies have passed an exhaustive process of validation and standardization for MRD testing in different hematologic malignancies, which make them readily available and reproducible among different centers (42, 43). However, these approaches require diagnostic samples with high tumor load to identify patient-specific clonotypic sequences (44, 45). Moreover, multiple myeloma is a postgerminal center neoplasia characterized by a high rate of somatic hypermutations both in the heavy- and light-chain immunoglobulin genes (46-48). Such mutations prevent the annealing of consensus primers, limit clonal detection, sequencing success rate and overall ASO performance, forcing the use of specific primers/probes instead (49). Consequently, it has been recently reported that the use of CD138$^+$ positively selected bone marrow PCs may significantly increase the applicability of PCR-based MRD studies in multiple myeloma (50). Nevertheless, the technique remains costly, laborious, and accordingly, difficult to implement into routine clinical practice.

Next-generation sequencing

The greatest advantage of NGS approaches for MRD detection in multiple myeloma is its sensitivity which, without compromising specificity, is estimated to be in the range of $10^{-5}$ to $10^{-8}$ (35, 51). Moreover, NGS can identify both stable and dynamic aspects of the BCR rearrangement, including potential clonal tiding (52). However, the presence of subclonality in diagnostic samples is typically below 7% of all patients and virtually impossible to be distinguished from biallelic rearrangements (53). Furthermore, the main clonal rearrangement is usually stable from diagnosis to relapse (N. Puig and colleagues; accepted for publication). Therefore, because clonal Ig gene sequences remain stable and because current MRD monitoring by ASO-PCR or NGS does not focus on gene mutations or copy-number abnormalities, molecular techniques are not influenced by genetic heterogeneity and clonal tiding throughout patients’ treatment. Other advantages that NGS offers are superior sensitivity, potential scalability, and less methodologic complexity. In particular, the latest advantage would be crucial in multiple myeloma because it removes the need of standard curve construction, which is the main reason of ASO-PCR failure in multiple myeloma (49). However, there are also some disadvantages. Molecular-based approaches cannot distinguish hemodiluted from good quality samples. Albeit the applicability of NGS is superior to that of ASO-PCR, still 10% of patients will be missed during the initial PCR step (51). In addition, MRD quantitation is only approximate, because the efficacy of amplification is highly variable depending on the specific sequence of the rearrangement (54). Because only B cells have rearranged immunoglobulins, NGS-based MRD monitoring will only quantify B cells; accordingly, global MRD quantitation requires the addition of an artificial internal control for such quantification. Finally, NGS is a labor-intensive and expensive technology, and it is yet not commonly available for clinical practice.

Clinical Results with (Intramedullary) MRD Techniques

Multiparameter flow cytometry

The prognostic value of MFC-based MRD monitoring in multiple myeloma was introduced in 2002 by San Miguel and colleagues (22) and Rawstron and colleagues (20), both studies suggesting the utility of monitoring the bone marrow PC compartment among multiple myeloma patients treated with conventional or high-dose chemotherapy, even if such patients were in CR (20). This initial positive experience led the Spanish and UK groups to implement their corresponding 4- and 6-color flow-MRD methods in large clinical trials. In the PETHERA/GEM2000 study, flow-MRD was identified as the most relevant prognostic factor in a series of 295 newly diagnosed multiple myeloma patients receiving uniform treatment including HDT/SCT (55). MRD negativity at day 100 after ASCI translated to significantly improved PFS and OS, and the impact of MRD was equally relevant among patients in CR. Similarly, in the intensive pathway of the MRC Myeloma IX study, MRD negativity at day 100 after ASCI was predictive of favorable PFS and OS (56). This outcome advantage was equally demonstrable in patients achieving CR. Because in multiple myeloma there has been extensive debate on whether attaining deep levels of remission (i.e., CR) would be critical to all patients or in turn is particularly relevant for patients with high-risk disease, it is important to emphasize that both the PETHERA/GEM and UK groups have demonstrated that risk assessment by FISH and flow-MRD monitoring were of independent prognostic value in transplant-eligible patients (55, 56). Furthermore, it is particularly interesting to observe the benefit of achieving MRD negativity in high-risk patients, whose outcome becomes similar to that of standard-risk patients (25). Accordingly, further research on the role of MRD as a surrogate for prolonged OS among high-risk patients is warranted, because it could represent an attractive clinical end-point to improve the overall poor prognosis of this patient population. Thus, combined cytogenetic/FISH evaluation at diagnosis plus MRD assessment after HDT/ASCT (day +100) provided powerful risk stratification, which also resulted in a highly effective approach to identify patients with unsustained CR and dismal outcomes (25). Collectively, these results confirm the superiority of MRD assessment over conventional response criteria to predict outcome in distinct multiple myeloma genetic subgroups. The effect of maintenance therapy with thalidomide was also assessed in the UK study. Interestingly, MRD-positive patients randomized to the maintenance arm experienced significantly prolonged PFS as compared with the placebo arm; in MRD-negative patients, a similar trend was observed (56). Further analyses by the PETHERA/GEM have shown that combining the prognostic information of cytogenetics at diagnosis plus MRD assessment at day 100 after HDT/ASCT resulted in a highly effective approach to identify patients with unsustained CR and dismal outcomes (2-year median OS); those with baseline high-risk cytogenetics plus persistent MRD after ASCT (57). The GEM has also shown that it was possible for elderly patients treated with bortezomb-based induction regimens to achieve MRD negativity, and that flow-MRD resulted in superior patient prognostication than conventional and stringent CR response criteria (7). More recently, the Intergroupe Franco- phone du Myélome has reported on the prognostic value of their 7-color flow-MRD method implemented in a recent phase II study (58). Overall, 68% of patients achieved MRD negativity and none
of these patients relapsed. Thus, it is plausible to assume that albeit the already well-established link between patients’ flow-MRD status and survival, the true clinical utility of flow-based MRD assessment is likely to be significantly improved with more sensitive \((10^{-3})\) and multidimensional \((\geq 8\)-color\) immunophenotyping. Table 1 summarizes the most relevant flow-MRD studies reported in multiple myeloma.

**Allele-specific oligonucleotide PCR**

A considerable number of studies have explored the value of PCR-based MRD monitoring in multiple myeloma (Table 2; refs. 59–65). Although initial observations lacked clinical value, additional studies performed in patients undergoing autologous or allogeneic SCI unnnerved the prognostic value of reaching molecular remissions (Table 2; refs. 39–41, 49, 50, 64, 66–71) Using nonquantitative approaches, the percentage of molecular remissions observed after allogeneic SCI was significantly higher as compared with patients undergoing autologous SCI, suggesting a role for this technique to evaluate treatment efficacy. Furthermore, Lipinski and colleagues, in a retrospective study performed in 13 patients undergoing ASCT, suggested the potential value of ASO-PCR monitoring to predict progression (72), and this notion of MRD reappearance heralding relapse has been recently confirmed by the GIMEMA group (71).

Semiquantitative and quantitative approaches have also been used to predict patients’ outcome according to MRD levels. Korthals and colleagues in a cohort of 53 patients undergoing ASCT have shown that different MRD levels by ASO-RQ-PCR before ASCT allowed to discriminate two groups of patients with different PFS and OS (41). Putkonen and colleagues in a series of 37 patients undergoing autologous and allogeneic stem cell transplantation defined 0.01% as the optimal MRD threshold to distinguish two groups of patients with different PFS and also OS (70). Puig and colleagues, in a recent study that included 103 patients undergoing ASCT, also found \(10^{-4}\) as the most significant cutoff level, distinguishing two subgroups with different PFS and, when applied to patients in conventional CR, also different OS (50). Finally, Ladetto and colleagues with nested and ASO-RQ-PCR have reported on the significant reduction of residual tumor load after bortezomib, thalidomide, and dexamethasone (VTD) consolidation, which translated into prolonged PFS (69). A recent update of the study showed that MRD monitoring also predicted for different OS: 72% at 8 years for patients in major MRD response versus 48% for those with positive MRD (71).

**Next-generation sequencing**

Because NGS-based MRD monitoring is still a relatively recent approach, there is yet few data in multiple myeloma. However, the PETHEMA/GEM has already described favorable and promising results in a series of 133 multiple myeloma patients, including both transplant and non–transplant-eligible cases. The applicability of NGS-based MRD monitoring using the Lympho-SIGHT methodology was of 90%. The median TTP and OS of MRD-negative cases were of 80 months and not reached, respectively (51). Importantly, Martinez-Lopez and colleagues (51) identified three groups of patients with different TTP: patients with high \((<10^{-3})\), intermediate \((10^{-3} \text{ to } 10^{-1})\), and low \((>10^{-1})\) MRD levels showed significantly different TTP: 27, 48, and 80 months, respectively, which indicates that the deepest the quality of CR, the better the patients outcome. Similar results

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**Table 1. Summary of the most relevant studies based on MFC detection of MRD in multiple myeloma**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Setting</th>
<th>Applicability</th>
<th>LOD</th>
<th>MOG</th>
<th>MOG according to MRD</th>
<th>PFS according to MRD</th>
<th>OS according to MRD</th>
<th>PFS according to MRD</th>
<th>OS according to MRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>San Miguel et al., 2002 (22)</td>
<td>8-color MFC</td>
<td>10^{-4} to 10^{-5}</td>
<td>94%</td>
<td>CT or CT</td>
<td>ASCT</td>
<td>43% (6/14)</td>
<td>96% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
<td>91% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rawstron et al., 2002 (20)</td>
<td>3-color MFC</td>
<td>10^{-4} to 10^{-5}</td>
<td>&lt;5%</td>
<td>ASCT</td>
<td>ASCT</td>
<td>74% (7/10)</td>
<td>96% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
<td>91% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Paiva et al., 2011 (7)</td>
<td>4-color MFC</td>
<td>10^{-4} to 10^{-5}</td>
<td>&lt;5%</td>
<td>VMP or VTP</td>
<td>ASCT</td>
<td>5% (5/102)</td>
<td>86% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
<td>91% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Roussel et al., 2014 (58)</td>
<td>5-color MFC</td>
<td>10^{-4} to 10^{-5}</td>
<td>&lt;5%</td>
<td>VRD</td>
<td>VRD</td>
<td>95% (154/261)</td>
<td>86% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
<td>91% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rawstron et al., 2007 (30)</td>
<td>6-color MFC</td>
<td>10^{-4} to 10^{-5}</td>
<td>&lt;5%</td>
<td>CT or CT</td>
<td>ASCT</td>
<td>5% (7/147)</td>
<td>86% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
<td>91% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rawstron et al., 2013 (49)</td>
<td>7-color MFC</td>
<td>10^{-4} to 10^{-5}</td>
<td>&lt;5%</td>
<td>CT or CT</td>
<td>ASCT</td>
<td>5% (154/261)</td>
<td>86% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
<td>91% vs. 70% at 3 years</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Abbreviations:** MOG, minimal residual disease; MFC, multiparameter flow cytometry; CT or CT, cyclophosphamide, vincristine, doxorubicin, and dexamethasone; CVAD, cyclophosphamide, vincristine, doxorubicin, and dexamethasone; ASCT, autologous stem cell transplantation; VTD, bortezomib, thalidomide, and dexamethasone; VTP, bortezomib, thalidomide, and prednisone; VMP, bortezomib, melphalan, and prednisone; VTP, bortezomib, thalidomide, and melphalan; VRD, bortezomib, lenalidomide, and dexamethasone.
Summary of the most relevant studies based on PCR detection of MRD in multiple myeloma

<table>
<thead>
<tr>
<th>Reference</th>
<th>Setting</th>
<th>Method</th>
<th>LOD</th>
<th>Applicability</th>
<th>Molecular remission</th>
<th>PFS according to MRD</th>
<th>OS according to MRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corradini et al., 2003</td>
<td>ALLO</td>
<td>ASO</td>
<td>4</td>
<td>NA</td>
<td>77%</td>
<td>0.001</td>
<td>NA</td>
</tr>
<tr>
<td>Bakkus et al., 2004</td>
<td>ASCT</td>
<td>ASO</td>
<td>3</td>
<td>NA</td>
<td>75%</td>
<td>0.04</td>
<td>0.008</td>
</tr>
<tr>
<td>Galimberti et al., 2005</td>
<td>ASCT</td>
<td>ASO</td>
<td>3</td>
<td>NA</td>
<td>86%</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>Putkonen et al., 2010</td>
<td>ASCT</td>
<td>ASO</td>
<td>3</td>
<td>NA</td>
<td>91%</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>Ferrero et al., 2014</td>
<td>ASCT</td>
<td>ASO</td>
<td>3</td>
<td>NA</td>
<td>91%</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>Puig et al., 2014</td>
<td>ASCT</td>
<td>ASO</td>
<td>3</td>
<td>NA</td>
<td>91%</td>
<td>0.001</td>
<td>0.002</td>
</tr>
</tbody>
</table>

NOTE: The low number of cases in most of the series is probably due to the technical problems related to this technique. However, in all studies the persistence of MRD was associated with a significantly shorter PFS and often OS as compared with MRD-negative patients. Discordant results from two molecular techniques that, albeit methodologically different, have similar sensitivity.

### Discordant Results among (Intramedullary) MRD Techniques

Multiparameter flow cytometry versus allele-specific oligonucleotide PCR

Three studies have directly compared MFC and ASO-PCR as alternative methods for MRD detection in multiple myeloma. In 2005, Sarasquete and colleagues quantified MRD levels in 24 patients at day 100 after HDT/ASCT using both techniques, and observed discordant results in 6 of the 24 cases; all of them negative by MFC but positive by ASO-RQ-PCR (39). Discordances were attributed to the limit of MRD detection by each technique, because the median number of clonal PCs in PCR+MRC- cases was 0.014%. A second study by Liozov and colleagues, performed on 69 samples from 13 patients undergoing allogeneic SCT, found a very high correlation between both techniques. Virtually, all results were concordant, and both techniques had similar applicability and prognostic value (73). More recently, Puig and colleagues compared the results of MRD assessment by MFC and ASO-PCR in 103 cases undergoing HDT/ASCT, and only observed 18 discordant results (11 cases were PCR+MFC- whereas the other 7 were MFC+ but PCR- ). No differences in survival were observed between the discordant groups of patients (49).

Multiparameter flow cytometry versus next-generation sequencing

Only one study has compared MFC versus NGS for MRD monitoring in multiple myeloma (51). From a total of 99 patients, 82 had concordant results (60 double-positive and 22 double-negative), 12 were MFC+-NGS-, and 5 were MFC-NGS+. Discordances are likely related to differences in sensitivity and specificity, which theoretically favor NGS over 4-color MFC. However, it cannot be excluded that certain clonal rearrangements could be under-amplified against polyclonal rearrangements and, if present in very low numbers (typical situation in MRD samples), these could remain undetectable. Noteworthy, the time to progression of MRD patients by NGS was slightly better than NGS+MFC- cases (P = 0.05). However, this study was a retrospective comparison, and the MFC approach was a conventional 4-color staining of a limited number of cells (10-fold less compared with current MFC studies; ref. 51).

Allele-specific oligonucleotide PCR versus next-generation sequencing

Only two studies have compared ASO-PCR versus NGS. Ladetto and colleagues have reported preliminary data on 10 multiple myeloma patients; 8 were evaluable by RQ-PCR, 8 by NGS, and 6 by both methods (74). Among total follow-up samples, 20 discordances were recorded: 12 qualitative and 8 quantitative discordances. In 9 samples, RQ-PCR yielded a positive or 1 log higher result compared with NGS, whereas the opposite occurred in 11 cases. Overall, major discordance rates in multiple myeloma were comparable to ALL and MCL, whereas minor and quantitative discordances appeared slightly more frequent (74). Martinez-Lopez and colleagues performed a similar comparison in 46 patients and observed a concordance rate of 85% (51). Thus, further studies are warranted to elucidate the clinical significance of discordant results from two molecular techniques that, albeit methodologically different, have similar sensitivity.

State of the Art: MRD Testing for Myeloma

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The ideal MRD test should fulfill a minimum of several relevant characteristics: (i) high applicability, (ii) high sensitivity, (iii) readiness and rapid turnaround, (iv) feasibility in low sample volumes, (v) reproducibility, and (vi) clinical utility. Because the cost of MRD assessment is significantly lower as compared with the cost of some drugs, its importance is less relevant; however, in the event of long-term follow-up sequential analysis (similarly to what is done in chronic myelogenous leukemia or acute lymphoblastic leukemia), total cost becomes significantly higher and therefore important to evaluate. Although we wait for further results on the comparison between ASO-PCR and NGS, the higher applicability of the latter would favor its incorporation in clinical trials. Unfortunately, data on prospective comparison between the next-generation MFC versus NGS is not yet available, and knowledge on head-to-head applicability and sensitivity is missing. Altogether, this precludes us to indicate at present a favorite methodology to be implemented in clinical trials. For routine clinical practice, we would support at this moment the use of next-generation MFC given its wide availability, cost effectiveness, and independence of previous collection of a diagnostic sample.

**Concluding Remarks and Future Directions**

Approximately 15 years after the first published results, MRD detection has emerged as one of the most relevant prognostic factors in multiple myeloma. Because MRD represents the collective end result of all of the cellular mechanisms that determine a patient response to a given therapy, MRD assessment affords prognostic information even among patients in CR, and irrespectively of disease risk. Accordingly, there is increasing interest on MRD monitoring as a sensitive tool to evaluate treatment efficacy, to become a surrogate marker for clinically relevant end-points, and for risk-adapted treatment, particularly in the consolidation and maintenance setting. However, the patchy pattern of bone marrow infiltration, typically observed in multiple myeloma, leads to some degree of uncertainty regarding an MRD-negative result irrespectively of the technique adopted: does it represent real absence of clonal PCRs, or is it due to sampling error? The possibility of patchy bone marrow infiltration or extramedullary involvement represents a challenge for both immunophenotypic and molecular-based MRD detection in single bone marrow aspirates. This highlights the value of sensitive imaging techniques to redefine CR both at the intramedullary (e.g., whole body MRI and PET/CT) and extramedullary levels (PET/CT). However, standardization of imaging techniques and comparison with other sensitive bone marrow–based MRD methods are still lacking. By contrast, the persistence of MRD is always an adverse prognostic feature, even among CR patients, envisioning that for the time being it would also be safer to take clinical decisions based on MRD positivity than on MRD negativity. The clinical applicability of MRD in multiple myeloma has been prospectively confirmed in two large transplant-based studies (55, 56) and a relatively smaller (yet prospective) clinical trial on transplant-ineligible multiple myeloma patients (7); such results were also retrospectively reproduced on smaller patient series mostly by using molecular techniques. In all studies, the PFS of MRD-negative patients at least doubled that of MRD-positve CR patients; conversely, both MFC and ASO-PCR showed that CR patients with persistent MRD had significantly inferior OS versus MRD-negative cases. MDR has also proven to be relevant in both standard- as well as high-risk patients. Altogether, these results strongly support the rationale for implementing MRD assessment to redefine and improve current CR criteria in multiple myeloma. The time has come to implement MRD monitoring in prospective clinical trials and fully establish its role in the management of multiple myeloma patients.

**Disclosure of Potential Conflicts of Interest**

B. Paiva reports receiving speakers bureau honoraria from Becton, Dickinson and Company; Binding Site, Celgene, Janssen, and Millennium Pharmaceuticals. J.F. San Miguel is a consultant/advisory board member for Bristol-Myers Squibb, Celgene, Janssen, Merck, Millennium Pharmaceuticals, Novartis, and Onyx Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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