Métodos para detectar la enfermedad mínima residual

Methods to detect minimal residual disease*

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Introduction

Minimal residual disease (MRD, also now called measurable residual disease in some publications) is of increasing importance in hematologic malignancies. It has been demonstrated to be an independent, post-diagnosis, prognostic indicator for several leukemias including AML and ALL and is of increasing and new importance for CLL and multiple myeloma (Table 1)(1-5). It is the backbone for treatment strategies in CML(6). Its results provide important information for risk stratification and treatment planning. MRD can be evaluated by multi-parameter flow-cytometry (MFC) and by molecular techniques. However, standardization, qualitatively as well as quantitatively, still needs a lot of efforts for broad application and routine clinical use in several diseases. Although many publications have proven the power of MRD measurement by either MFC or molecular techniques, before or even after allogeneic stem cell transplantation (SCT), additional studies implementing this tools for adapted treatment strategies are warranted. No new study should start without at least the option to study MRD.

Current state-of-the-art

• Overview

The diagnostics and treatment options of hematological malignancies have been conjointly optimized over the last decades. Improved and novel treatments lead to deeper responses and to better patient outcome, and provide challenges in measuring said responses. This encouraging development has led to a diagnostic shift: from phenotype to genotype. While morphology-based methods have a sensitivity of 1:20(3), the current state-of-the-art
techniques for assessment of residual disease, MFC or molecular methods, demonstrate a sensitivity of $1:10^4$ to $1:10^6$ and thus also supersede chromosome analysis and FISH.

Several approaches are used and have been published to definitely detect the malignant cells. In MFC there are in general two strategies for MRD assessment: the leukemia associated immunophenotype (LAIP) and the different-from-normal-approach (DfN). For molecular techniques there is an even broader method diversity. While the current gold standard is the real-time quantitative polymerase chain reaction (qPCR), newer techniques are being tested for broad clinical applications such as digital PCR (dPCR) or next generation sequencing (NGS)\(^\text{(1)}\).

Harmonization of protocols as well as of antibody combinations for MFC can be implemented. Individual, laboratory based strategies, however, still are much more often applied.

**Multi-parameter flow-cytometry**

One of the two approaches of MRD analysis by MFC relies on the detection and knowledge of the respective character of the leukemic cells at diagnosis, leading to the definition of the so called “leukemia associated immunophenotype (LAIP)”, while the “different-from-normal-approach” relies on the knowledge of normal bone marrow and differences from this detected at the time of MRD assessment. Aberrant immunophenotypes can be categorized into\(^\text{(7)}\):

- Cross-lineage antigen expression (e.g. expression of lymphoid markers in AML (such as CD19+ AML))
- Maturational asynchronous antigen expression (i.e. concomitant expression of immature and mature markers (e.g. coexpression of CD34 and CD11b in AML))
- Reduction or absence of typical markers (e.g. HLA-DR-negative AML)
- Antigen overexpression (e.g. CD33++ CD34++ AML)

Different panels at diagnosis have to be used for the respective disease entities and normally include several additional, specific CD markers in addition to the mandatory markers for diagnosis. This leads to a much higher sensitivity and specificity for follow-up investigations. In acute leukemias also several different combinations of CD markers can lead to more than one LAIP, even increasing sensitivity. Based on the definition of the specific characteristics of these specific leukemia cells of the individual patient, MRD by MFC, at least for acute leukemias should be based on a minimum of 8 colors, and can widely be used in dedicated laboratories. Several guidelines have been published in the last years to support antibody selection, read out and reporting\(^\text{(6,9)}\). If possible, peripheral blood and bone marrow should be investigated at diagnosis, both materials could also be used at follow-up. Samples can be analyzed up to 72 hours after drawing, however, shorter travel times should be preferred. Standardization of gating as well as analysis and report should be improved. If possible, MRD studies by MFC should be implemented in all future clinical studies, especially to foster post-remission treatment strategies, proactively including or excluding transplantation for the respective patient based on his MRD level\(^\text{(2,10-16)}\).

**Molecular techniques**

Increasing efforts have been undertaken to standardize molecular techniques for MRD, especially in CML by introducing the International Scale (IS) and standardized ring trials. Several ELN guidelines have been demonstrated to serve as best treatment stratification information\(^\text{(6,17)}\). Also in ALL, post-remission MRD has proven to be of important clinical relevance. Due to the increasing knowledge about molecular markers in hematology new targets for MRD have been detected and need further clinical evaluation. The sensitivity of molecular methods is in most cases comparable to MFC, in some diseases or in comparison to some antibody-combinations vs. molecular markers the one or the other technique is superior and has to be defined for the individual patient and its leukemic cells at diagnosis in comparison.

(Potential) molecular MRD markers can be divided into four different categories\(^\text{(1,2)}\):

- fusion gene transcripts (e.g. BCR-ABL1)
- gene mutations (e.g. mutated NPM1 in AML)
- gene rearrangements (e.g. immunoglobulin/T cell receptor gene rearrangements for MRD assessment in lymphoid neoplasms)
- gene expression levels (e.g. WT1 in AML)

For MRD diagnostics the most relevant molecular
methods are PCR-based techniques and next generation sequencing. Quantitative real-time PCR (qPCR) currently represents the gold standard in routine MRD diagnostics due to its high sensitivity, which varies between 0.001% and 0.1% mutational load, depending on the respective mutation. Allele-specific qPCR strategies enable the monitoring of mutations, while fusion gene transcripts as well as gene expression can be quantified by qPCR, after transcripts are reverse transcribed (RT) into cDNA (RT-qPCR).

While qPCR quantification relies on standard curves (relative quantification), a new and innovative PCR technique, called digital PCR allows for absolute quantification of target sequences. The sample is compartmentalized into smaller PCR reaction volumes, some of which contain no template molecules. In contrast to qPCR, the PCR reaction is not followed in real-time. Instead fluorescence readout is performed after endpoint PCR. The readout is binary (signal or no signal). To account for PCR reaction volumes that contained more than one target molecule, a poisson correction factor is applied, when summing up the signals to allow for correct absolute quantification (18).

Next generation sequencing is replacing the older Sanger sequencing technique more and more in the clinical routine. Sanger sequencing has a sensitivity of 10-20% mutational load and is therefore not suited for MRD diagnostics. Aside to the increased sensitivity of 1-3% mutational load, next generation sequencing allows for massive parallel sequencing. Panel based sequencing, for example, permits to analyze the mutational status of all known leukemia-associated genes. To improve sequencing depth, relevant gene segments are enriched prior to next generation sequencing either by targeted enrichment or amplicon deep sequencing.

New NGS applications are whole genome sequencing and whole exome sequencing, providing insight into molecular aberrations throughout the whole genome, or all protein coding genes, respectively. In addition to sequence variations, these techniques can also detect balanced gene rearrangements (potentially resulting in fusion genes) as well as chromosomal gain and losses.

In the context of MRD diagnostics, NGS is currently most valuable for evaluation of the molecular landscape and the identification of (potential) markers for MRD. New and innovative strategies, such as the use of optimized polymerases for enrichment as well as the application of “unique molecular identifiers” together with improved bioinformatics analysis will pave the way for NGS as a method not to only identify, but to also sensitively monitor MRD markers (19).

**Outlook**

As many new molecular markers have been described in the last years, several prospective studies should be initiated to define and validate the best marker and its clinical relevance in MRD post-remission, for guiding indication to transplant as well as for post-transplant follow-up. In addition to RT-PCR, new techniques such as digital PCR and NGS based assays will show their clinical relevance in the next years to define not only the best MRD marker by molecular approaches, but also the respective most sensitive and reliable technique. Further guidelines in addition to those already published especially for CML and ALL but also for some markers in AML such as NPM1, are needed as well as bioinformatic support and definitions how to report results and how often and when MRD should be investigated. This is true for molecular techniques as well as for MFC. A recent review for AML summarizes state-of-the-art (1).

**Implementation of MRD techniques**

Table 1 gives an overview on which method is best suited to measure residual disease depending on the disease entity. Further details about the implementation and clinical utility are given in the following paragraphs.

**AML**

**Implementation of MRD methods in AML.**

Whether the MRD status in AML is best determined by multi-color flow-cytometry or by molecular techniques depends on the AML subtype and/or the individual case. On the one hand, there are 11 genetically defined AML entities. Also there is a high incidence of molecular mutations in AML: in 96% of patients at least one driver mutation is detectable (20). On the other hand, not every mutation is a suitable marker, whether due to insufficient sensitivity in detection or for biological reasons. Mutations in CEBPA, RUNX1, DDX41, GATA2 and ANKRD26 might
be present in the germline and their somatic nature has to be confirmed prior to MRD assessment\(^{(1)}\). For the mutations: FLT3-ITD, FLT3-TKD, NRAS, KRAS, IDH1, IDH2, MLL-PTD, there is a high probability of losses or amplifications upon relapse, thus those should not be used as sole MRD markers\(^{(1)}\). Mutations in ASXL1, TET2 and DNMT3A are associated with age dependent clonal hematopoeisis and often persist during AML treatment, therefore they should not be considered as suitable MRD markers. Expression level analysis of WT1 and EVI1 are also not recommended by the European LeukemiaNet MRD Working Party as (sole) MRD markers\(^{(1)}\).

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For both methods, however, standardization is necessary to improve and harmonize MRD diagnostics and to establish clear criteria for AML management\(^{(1)}\).

**Clinical value of MRD assessment in AML.** Independent from the method used to detect MRD, MRD positivity in AML is associated with increased relapse risk and reduced survival\(^{(1,21-23)}\). In some cases, MRD detection already serves as valuable decision making tool in AML. Pre-emptive arsenic trioxide therapy reduced the relapse risk of MRD positive patients with APL (PML-RARA)\(^{(24)}\).

Pre-emptive therapy using donor lymphocyte infusions (DLI) \(\pm\) hypomethylating agents is indicated when MRD positivity is detected in patients that underwent allogeneic transplantation\(^{(25)}\). In addition, there is evidence that stratification of patients with AML with t(8;21)/RUNX1-RUNXIT1 or with mutated NPM1 based on their MRD status after induction and consolidation therapy can identify those most likely to benefit from allogeneic stem cell transplantation\(^{(26)}\) and/or therapy with high dose cytarabine\(^{(27,29)}\).

### Table 1. Methods to detect MRD according to disease entities

<table>
<thead>
<tr>
<th>Method (MRD)</th>
<th>Sensitivity</th>
<th>AML</th>
<th>ALL</th>
<th>CML</th>
<th>CLL</th>
<th>Myeloma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multi-parameter flow-cytometry</td>
<td>1:10(^4)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1:10(^6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular approaches</td>
<td>1:10(^4)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>1:10(^6)</td>
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</tbody>
</table>

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**Clinical value of MRD assessment in ALL.** The ideal method for MRD assessment has to be determined individually. Several ALL subtypes are genetically defined by a pathogenic fusion gene (e.g. **BCR-ABL1** positive ALL). The expression of said fusion genes can be measured by RT-qPCR and fusion transcripts represent sensitive and reliable MRD markers. In the absence of fusion genes, the detection and quantification of patient-specific clonal rearrangements of immunoglobulin genes or T cell receptor genes provides another possibility for molecular MRD assessment, and is applicable for >95% of patients\(^{(2)}\). It has been shown for **BCR-ABL1** positive ALL that the results from both molecular methods complement each other\(^{(30,31)}\), thus, if applicable, the parallel use of both strategies might provide valuable information. In addition, minimal residual disease in ALL can also be assessed by MFC.

**Clinical value of MRD assessment in AML.** MRD has a well-established role in adult ALL as the strongest independent prognostic factor at any time during or after treatment\(^{(2)}\). MRD assessment even allows for patient stratification, as patients who achieve an early molecular complete remission during induction therapy (defined as MRD negativity) have a very favorable prognosis\(^{(32)}\). In contrast,
persistent MRD positivity or molecular relapse after consolidation phase 1 are associated with a higher risk of relapse\(^{(33)}\). While these criteria identify a subgroup of patients that profit from SCT\(^{(33)}\), there are studies that show that pre-SCT MRD status and disease kinetics still retain their prognostic value in the SCT setting\(^{(34,35)}\).

Currently, post-SCT relapse detection is routinely performed by chimerism analysis. Studies that evaluate the clinical utility of MRD assessment post-SCT are few, however, they show that MRD status correlated with outcome and relapse risk\(^{(36,37)}\). In addition, MRD may be able to detect early relapses earlier and in a more specific manner than chimerism analysis\(^{(38)}\).

• **CML**

CML is a model case for a genetically defined disease entity and highlights how improved treatment (by the introduction of tyrosine kinase inhibitors (TKI)), results in better and deeper responses, and thus also warrants advances in capturing residual disease.

**Implementation of MRD methods in CML.** Assessment of response and MRD in CML relies strongly on the measurement of \(BCR-ABL1\) transcript levels using reverse transcription quantitative PCR (RT-qPCR) to determine \(BCR-ABL1\) transcript levels. Due to standardization and introduction of the International Scale (IS), a harmonized definition of molecular responses now guides CML management.

**Clinical value of MRD assessment in CML.** A 3-log reduction of \(BCR-ABL1\) (\(BCR-ABL1^{IS} \leq 0.1\%\)) transcript levels is considered a major molecular response (MMR), and should be achieved within 12 months of TKI therapy start. A “warning” is issued, if transcript levels are between 0.1 and 1\%, \(BCR-ABL1^{IS}\) levels above 1\% constitute a “failure”. Loss of MMR (in two consecutive tests) at any time is also considered a therapy failure, and should trigger \(BCR-ABL1\) mutational testing and change of TKI\(^{(6)}\).

Given the high sensitivity of RT-qPCR, “deep” molecular responses (MR) have been defined and are indicated by the log reduction in uppercase (e.g. MR\(^{4.5}\)). Such deep molecular responses in CML have served as a study end point in the evaluation of nilotinib\(^{(39)}\) and are a prerequisite for participation in a TKI stop study\(^{(40)}\).

• **CLL**

**Implementation of MRD methods in CLL.** MRD in CLL can be monitored either by MFC or molecular techniques, such as allele-specific oligonucleotide PCR or a next generation sequencing approach\(^{(41)}\). Both molecular methods assess the individual clonal IG-rearrangement, its determination at time of diagnosis is a prerequisite for subsequent response and MRD monitoring\(^{(42)}\). Blood sampling for MRD detection might be prone to artifacts, since certain therapies (e.g. monoclonal antibodies) preferentially lead to clearance in the blood, but not in the bone marrow. In such cases, additional marrow samples may be important for determination of a reliable MRD status\(^{(41)}\).

**Clinical value of MRD assessment in CLL.** In CLL, MRD status is a valuable prognostic indicator after chemotherapy and MRD positivity is associated with a decrease in progression free survival (PFS) and overall survival (OS)\(^{(43-46)}\). Currently, the MRD status has no clinical implications, however, there is discussion as to whether therapy decisions should be based on MRD assessment\(^{(42)}\).

• **Myeloma**

**Implementation of MRD methods in multiple myeloma.** In the past, the main approach to measure residual disease in multiple myeloma was the quantification of monoclonal protein or the detection of residual myeloma cells by cytological assessment of bone marrow\(^{(47)}\). However, as of recently, modern flow-cytometry and/or next generation sequencing enable the detection of a residual plasma cell in up to \(10^6\) bone marrow cells and allow for sensitive MRD detection in multiple myeloma\(^{(48,49)}\). Imaging techniques should complement MRD assessment, since bone marrow based MRD examinations are not able to detect extramedullary disease\(^{(47)}\). The response criteria of the International Myeloma Working Group (IMWG) accordingly distinguish between different types of MRD negativity: *Flow MRD negativity* (absence of immunophenotypically aberrant plasma cells in bone marrow aspirates), *Sequencing MRD negativity* (absence of clonal plasma cells in
bone marrow aspirates detected by NGS) and Imaging MRD negativity (MRD negativity demonstrated by MFC or NGS AND absence of residual disease in PET-CT imaging(49).

Clinical value of MRD assessment in multiple myeloma. MRD negativity is associated with prolonged survival (PFS and OS)(48,50). Despite its prognostic importance, MRD diagnostics has no clinical relevance for multiple myeloma as of yet. However, MRD detection is discussed or used as study end point(47,51). Further studies have to elucidate whether MRD triggered therapy improves patient outcome(47,52).

Future perspectives
MRD by immunophenotyping or by molecular techniques will gain much more influence in post-remission strategies for hematological malignancies in the next years. Both techniques have advantages as well as disadvantages, however, combining (if applicable) both approaches might improve prognostic evaluation. This notion is underlined by a recent case study in AML. The concomitant use of molecular and MFC based MRD detection identified a higher proportion of patients that are at risk of relapse than either one method. Concordant results were obtained for 69.1% of the patients. In cases where MRD positivity was demonstrated by MFC and molecular testing, the relapse risk was 73.3%. In comparison, if MRD positivity was detected solely by either one method the relapse risk was ~50%(53). In the study by Jongen-Lavrencic and colleagues molecular MRD markers were identified by panel based NGS testing, with a panel comprised of 54 genes associated with myeloid neoplasms. With the exception of mutations in DNMT3A, TET2 and ASXL1, which are associated with age related clonal hematopoeisis, detection of any mutation served as suitable marker for MRD assessment, with high prognostic relevance for the estimation of relapse risk and survival(53).

In multiple myeloma a small study for the validation of NGS based determination of the IGH “clonotype” compared this novel approach to MFC for 66 patients. Results were concordant in 89%. However, patients with discordant results (MRD positivity detected by either method) had an intermediate progression free survival (of 46 months) in comparison to a PFS of 32 months for concordant MRD detection. This end point was not reached for cases with “double-negative” MRD(54).

In the years to come, our knowledge on how to stratify patients to the best MRD technique(s) to monitor response and residual disease during treatment and in remission will continue to grow. Concomitantly, with clear definition of response and MRD criteria (as in CML), MRD assessment might also gain more importance as a clinical decision making tool. It may facilitate the clinical decision to transplant or not to transplant in first CR or in later CR. Further, therapeutic approaches after allogeneic transplantation such as the use of DLI, can be guided by MRD. Further, as demonstrated by CML already, MRD levels can lead to stop treatment or avoid further chemotherapy, which automatically reduces side effects for the patient.

It is foreseeable that the use and the clinical importance of MRD studies, in clinical trials but also in daily practice, will not only lead to more specific, individualized treatment and patient follow-up, but also reduce toxicity and costs. Therefore, efforts should be taken much more to define the respective and best technique to use for MRD to improve patients care and also to reduce costs in hematology in the next years.

The advance of whole exome and whole genome sequencing will also help to expand our knowledge on mutational landscape and identify new potential markers for MRD assessment. For lymphoid diseases without bone marrow involvement, liquid biopsy might enable molecular detection of MRD in the future based on samples of peripheral blood – and with higher sensitivities than the currently used imaging techniques(55).

Recommended readings
State-of-the-art review on MRD in AML.

State-of-the-art review on MRD in ALL.

Recommendations on MRD in CML.

MRD to guide therapy in AML.

Treatment relevance in ALL based on MRD.

Conflicto de interes: El Dr Torsten Haferlach declara haber recibido honorarios por parte de Illumina por concepto de conferencias y actividades educativas en las que ha participado. La Dra Ines Schmidts declara no poseer conflictos de interés.

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