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Special Report

2021 Update on MRD in acute myeloid leukemia: a consensus document from the European LeukemiaNet MRD Working Party

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Measurable residual disease (MRD) is an important biomarker in acute myeloid leukemia (AML) that is used for prognostic, predictive, monitoring, and efficacy-response assessments. The European LeukemiaNet (ELN) MRD Working Party evaluated standardization and harmonization of MRD in an ongoing manner and has updated the 2018 ELN MRD recommendations based on significant developments in the field. New and revised recommendations were established during in-person and online meetings, and a 2-stage Delphi poll was conducted to optimize consensus. All recommendations are graded by levels of evidence and agreement. Major changes include technical specifications for next-generation sequencing-based MRD testing and integrative assessments of MRD irrespective of technology. Other topics include use of MRD as a prognostic and surrogate end point for drug testing; selection of the technique, material, and appropriate time points for MRD assessment; and clinical implications of MRD assessment. In addition to technical recommendations for flow- and molecular-MRD analysis, we provide MRD thresholds and define MRD response, and detail how MRD results should be reported and combined if several techniques are used. MRD assessment in AML is complex and clinically relevant, and standardized approaches to application, interpretation, technical conduct, and reporting are of critical importance.

Introduction

Assessment of measurable residual disease (MRD) in acute myeloid leukemia (AML) is challenging. Several technologies are available for MRD quantification, but the assays and reporting lack standardization and comparability. Still, detection of MRD by any methodology during morphological remission after standard chemotherapy is a strong prognostic factor for subsequent relapse and shorter survival in patients with AML.[1] MRD monitoring may have value in guiding postremission therapy and identifying early relapse and as a surrogate end point in clinical trials to accelerate development of novel regimens. MRD assessment in AML has elicited considerable interest from clinicians, patients, regulatory authorities, industry, and researchers, and guidance in harmonization, refinement, and validation of MRD testing is needed.

Table 1. ELN 2021 MFC-MRD recommendations based on a Delphi poll

No.	Multiparameter flow cytometry MRD recommendation	LoE	GoR	LoA (%)
A1	When available, a diagnostic sample is preferred to: 1. determine if a patient has a diagnostic flow cytometric MRD target, and 2. assess treatment efficacy on potential clearance of the diagnostic LAIP populations.	V	B	94
A2	Implementation of a minimum required set of tubes/ fluorochromes combination is a prerequisite for harmonized LAIP/DfN MRD detection, analysis and reporting.	I	A	94
A3	We recommend harmonized use of the integrated diagnostic LAIP and DfN strategy for MRD detection that incorporates the core MRD markers CD34, CD117, CD45, CD33, CD13, CD56, CD7, and HLA-DR, to assess all samples.	V	B	88
A4	Particular attention should be devoted to evaluating the expression of the identified aberrant immunophenotypes in control samples that include regenerating BM.	V	A	88
A5	When immunophenotypic abnormalities in specific samples may reflect transient features of regenerating or "stressed" hematopoiesis, the MRD report should comment on this possibility and note that a repeat sample in 2-4 wk, if clinically indicated, may be informative.	V	C	94
A6	Request first-pull BM aspirate for MRD, and process sample within 3 days of storage, undiluted, in ambient conditions.	V	A	94
A7	For samples stored at ambient temperature >3 d, the MRD report should make specific note of potentially compromised cell viability.	V	B	94
A8	Explore strategies to assess hemodilution that can be incorporated and reported as part of the MRD assay.	V	B	88
A9	For clinical decision making, MRD assessment should be performed with a qualified assay, as based on the guidelines for rare events in MFC.	I	A	76
A10	To ensure the best quality of relevant events acquisition, use a gating syntax including FSC vs time and doublet exclusion plots.	V	A	81
A11	The standard for determining MFC MRD negativity is the acquisition of >500 000 $CD45^+$ cells and ≥100 viable cells in the blast compartment assessed for the best aberrancy(ies) available.	V	B	76
A12	LLOD and LLOQ should be calculated to assess MFC-MRD assay performance.	V	B	93

GoR, grade of recommendation; LLOD, lower limit of detection; LLOQ, lower limit of quantification; LoA, level of agreement; LoE, level of evidence.

The goal of the European LeukemiaNet (ELN) AML MRD expert panel was to update our previous consensus article and provide our latest insights and expert recommendations on different technologies and current clinical uses of MRD.[2] The updated guidelines were written according to consensus achieved by using a Delphi poll (supplemental Methods and supplemental Table 1, available on the *Blood* Web site) and the overall results are summarized in Tables 1 to 4.[3]

Since the 2018 guidelines,[2] we have replaced the term "minimal residual disease" with "measurable residual disease." A "positive" or "negative" MRD test result refers to the detection, or not, of measurable disease above specific thresholds that may vary by assay and by laboratory. Clinicians are advised to clarify the interpretation of individual MRD results with their MRD laboratory colleagues. It is important to recognize that a negative MRD result does not necessarily indicate disease eradication but, rather, represents disease below the assay's threshold in the tested sample, and patients may still experience relapse. Also, an MRD assay with a nonzero result may still be called "negative" by a laboratory if the level detected is below the threshold linked to prognosis.

Technologies

Multiparametric flow cytometry-MRD testing

Immunophenotyping is an essential, readily available tool for diagnosing AML and is currently the most commonly used MRD detection methodology. Supplemental Table 2 summarizes recent clinical studies incorporating multiparameter flow cytometry (MFC)-MRD assessment in AML, including for randomized treatment comparisons[4,5] and MRD-directed therapy.[6,7] Herein, we update current best practices (Table 1). Our consensus

Table 2. ELN 2021 molecular MRD recommendations based on a Delphi poll

No.	Molecular MRD recommendation	LoE	GoR	LoA (%)
B1	Techniques for molecular MRD assessment should reach an LOD of 10^{-3} or lower. To achieve this LOD, qPCR, dPCR, or error-corrected NGS using UMIs is recommended.	IV	B	100
B2	Either EDTA or heparin can be used on samples as an anticoagulant for molecular MRD analysis.	V	C	76
B3	Only 5 mL of BM aspirate should be used for molecular MRD assessment from the first pull (or the first pull after repositioning, if the initial pull is used for flow-MRD).	V	B	94
B4	The method of cell isolation should be kept consistent, as it may alter the leukemic cell percentage (eg, Ficoll separation to reduce dilution of leukemic cells with normal granulocytes or lysis of whole blood).	V	B	82
B5	Leukemia-specific PCR assays (eg, for *NPM1*, *PML-RARA*, or *CBF* AML) are preferred over fewer specific markers, such as *WT1* or *EVI1* expression.	V	B	78
B6	Targeted NGS-MRD using specific mutations identified at diagnosis vs agnostic panel approaches has different strengths and limitations, but both approaches can be considered, depending on sensitivity, turnaround time, resource use, setting (research, clinical trial, or clinical routine), and ability to standardize methodology and reporting.	IV	B	88
B7	If a panel approach is used for NGS-MRD, emerging variants not found at diagnosis should be reported only if confidently detected above background noise.	IV	B	89
B8	For NGS-MRD, we recommend considering all detected mutations as potential MRD markers, with the limitations detailed in recommendations B9 to B11.	IV	B	100
B9	Germline mutations (VAF of ~50 in genes *ANKRD26*, *CEBPA*, *DDX41*, *ETV6*, *GATA2*, *RUNX1*, and *TP53*) should be excluded as NGS-MRD markers, as they are noninformative for MRD.	V	A	94
B10	Mutations in *DNMT3A*, *TET2*, and *ASXL1* (DTA) can be found in age-related clonal hematopoiesis and should be excluded from MRD analysis.	IV	A	100
B11	Mutations in signaling pathway genes (*FLT3-ITD*, *FLT3-TKD*, *KIT*, and *RAS*, among others) most likely represent residual AML when detected, but are often subclonal and have a low negative predictive value. These mutations are best used in combination with additional MRD markers.	IV	B	94
B12	NGS-MRD analysis in patients treated with targeted agents (FLT3 inhibitors and IDH1/IDH2 inhibitors) should include the molecular marker that is targeted, but also others that are present in the sample.	V	A	94
B13	As of this writing, there is no uniform bioinformatics pipeline/platform for NGS-MRD variant calling. Harmonization efforts are strongly recommended, preferably using published open-source algorithms.	V	A	94
B14	Potential cross-sample sequence contamination as a result of pooling samples in NGS-MRD should be bioinformatically evaluated.	V	A	100

GoR, grade of recommendation; LoA, level of agreement; LOD, limit of detection; LoE, level of evidence; UMI, unique molecular identifier.

recommendations for optimized technical requirements for MFC-MRD is described in a separate publication.[8]

Leukemia-associated immunophenotype and difference from normal The flow cytometry expert panel continues to recommend integration of diagnostic leukemia-associated immunophenotype (LAIP) and different from normal (DfN) aberrant immunophenotype approaches to enable tracking of diagnostic and emergent leukemic clones. Both approaches require expertise in the recognition of aberrant populations and exclusion of potential background as part of assay validation. Ideally, a diagnostic sample is preferred to determine whether a patient has diagnostic flow cytometric MRD targets that can be tracked (recommendation A1). Group A recommendations are shown in Table 1. Implementation of a common, minimum required set of tubes/fluorochromes is a prerequisite for harmonized MRD

Table 3. ELN 2021 future improvement of MRD recommendations based on a Delphi poll

No.	Future improvement of MRD recommendation	LoE	GoR	LoA (%)
C1	LSCs can be immunophenotypically defined as $CD34^+/CD38^-$ cells[55] combined with an aberrant marker not present on HSCs (eg, CD45RA, CLL-1, or CD123).	IV	A	95
C2	Measurements of LSCs may have prognostic value and should be further validated in prospective clinical trials.	IV	B	86
C3	LSC detection requires optimally 4 million events, most likely best achieved with a 1-tube assay.	V	B	78
C4	High-quality flow cytometry data (standardized instrument settings, preanalytics, and measurements) are necessary for future automated analyses.	IV	A	100

GoR, grade of recommendation; LoA, level of agreement; LoE, level of evidence.

detection, analysis, and reporting (recommendation A2). We recommend harmonized use of the integrated diagnostic-LAIP and DfN strategy for MRD detection that incorporates core MRD markers CD34, CD117, CD45, CD33, CD13, CD56, CD7, and HLA-DR to assess all samples (recommendation A3). Some investigators favor addition of CD38 whenever possible, as CD38 adds specificity to certain aberrant leukemic immunophenotypes, particularly for the $CD34^+CD38^{low-}$ compartment, when markers such as CD56, CD7, and others, such as CD45RA, designated as leukemic stem cell markers, are aberrantly expressed. In cases with a monocytic component, additional markers (eg, CD64, CD11b, and CD4) may also be relevant.[9] The DfN approach detects aberrant clones regardless of immunophenotypic shifts, because it does not rely on the stability of a diagnostic LAIP during treatment, but defines "empty spaces" not occupied by cells within the normal differentiation profiles of bone marrow (BM) or peripheral blood (PB).[10] The panel advises the combined LAIP/DfN approach, but notes that some abnormal immunophenotypes may appear and/or disappear during monitoring, potentially because of transient expression on regenerating nonleukemic progenitors.[11-13] This phenomenon may affect the respective specificities of both LAIP and DfN MRD detection, in particular when the percentages of LAIPs at lower thresholds (eg, <0.1%) are investigated. Particular attention should be devoted to evaluating expression of the identified aberrant immunophenotypes in control samples that include regenerating BM (recommendation A4). When immunophenotypic abnormalities in specific samples could reflect transient features of regenerating or stressed hematopoiesis, the MRD report should comment on this possibility and note that a repeat sample in 2 to 4 weeks, if clinically indicated, may be informative (recommendation A5).

Sampling and preanalytical phase: technical requirements The panel strongly recommends submitting the first pull of BM aspirate for MRD analysis, as sample quality is critical for accurate results.[14] The sample should be processed undiluted within 3 days of storage at ambient conditions (recommendation A6). For samples stored at ambient temperature >3 days, the MRD report should make specific note of sample quality and potentially compromised cell viability (recommendation A7).

Sample preparation can be performed using 2 accepted techniques: (1) bulk lysis, followed by wash/stain/wash; or (2) stain/lyse/wash or no wash.[10,15] The technique selected should reliably produce high-quality MFC measurements (ie, optimal cell concentration and no loss of forward scatter [FSC] or side scatter [SSC] properties) and should be applied consistently across samples.

Basic principles of instrument settings are described elsewhere, and we suggest using standard operating procedures developed by international flow cytometry consortia.[16,17] Also, efforts should be made to evaluate sample quality with respect to PB contamination.[18,19] In general, our recommendation is for each laboratory to explore strategies to assess hemodilution that can be incorporated and reported as part of the MRD assay (recommendation A8).

Gating strategies and calculations for MFC-MRD MFC-MRD assessment used for clinical decision making should be performed with a qualified assay, as based on the guidelines for rare events in MFC (recommendation A9).[20-23] Acquisition should collect the highest possible number of relevant events and, accordingly, to ensure quality of relevant events acquisition, use a gating syntax including FSC vs time and doublet exclusion plots (eg, FSC-area vs FSC-height) (recommendation A10). Viability can be assessed by the addition of a viability dye or simply by accurate gating based on physical parameters (low FSC vs low SSC). As with the previous guidelines, the recommendation remains that the standard for determining MFC MRD negativity is to acquire >500 000 CD45 expressing cells and at least 100 viable cells in the blast compartment assessed for the best aberrancy(ies) available (recommendation A11).

To reliably use flow MRD for clinical decision making, studies of the lower limit of detection (LLOD) and lower limit of quantification (LLOQ) are essential. Thus, the panel recommends that LLOD and LLOQ should be calculated to assess MFC-MRD assay performance (recommendation A12) for each panel combination used. This statement aligns with the advice of regulatory agencies, which emphasizes that reporting MRD^- results without LLOD information is not meaningful.[24] The expert panel recognizes the complexity of MFC-based MRD in AML, where each LAIP may have its own background noise that could individually affect LLOD and LLOQ.[25]

Table 4. ELN 2021 clinical MRD recommendations based on a Delphi poll

No.	Clinical MRD recommendation	LoE	GoR	LoA (%)
D1	MRD should be assessed to refine relapse risk in patients who achieve morphologic remission, with full or partial hematologic recovery (CR/CR_i/CR_p/CR_h).	I	A	89
D2	For patients with mutant *NPM1*, CBF AML (*RUNX1-RUNX1T1* or *CBFB-MYH11*), or APL (*PML-RARA*), we recommend molecular MRD assessment by qPCR or dPCR.	II	A	88
D3	AML patients who are not included in the molecularly defined subgroups should be monitored for MRD by MFC.	II	A	84
D4	NGS-MRD monitoring is useful to refine prognosis in addition to MFC but, to date, there are insufficient data to recommend NGS-MRD as a stand-alone technique.	IV	B	84
D5	In *NPM1*-mutated AML, MRD should be assessed preferentially in PB after 2 cycles of chemotherapy, in BM at the end of consolidation, and in BM every 3 mo for 24 mo after the end of consolidation. Alternatively, MRD may be assessed from PB every 4 to 6 wk during follow-up for 24 mo.	IV	B	95
D6	In *RUNX1-RUNX1T1*, and *CBFB-MYH11* mutated AML MRD should be assessed preferentially in PB after 2 cycles of chemotherapy, in BM at the end of consolidation treatment, and in PB every 4 to 6 wk for 24 mo after the end of consolidation.	IV	B	94
D7	In APL, the most important MRD end point is PCR negativity for *PML-RARA* at the end of consolidation.	I	A	100
D8	For patients with non–high-risk APL, MRD monitoring is recommended only after completion of consolidation and may be discontinued once BM MRD negativity is achieved.	V	B	100
D8a*	For high-risk APL MRD should be assessed by qPCR from BM every 3 mo for 24 months starting at the end of treatment. Alternatively, MRD may be assessed from PB every 4 to 6 wk during follow-up.	—*	—	—
D9	Ongoing molecular MRD monitoring beyond 24 mo of follow-up should be based on individual clinical features.	V	C	95
D10	Patients who are followed-up with MFC-MRD should have BM assessment after 2 cycles of chemotherapy, at the end of consolidation, and prior to stem cell transplantation, if applicable.	II	A	100
D11	MFC-MRD test positivity is defined as ≥0.1% of CD45-expressing cells with the target immunophenotype.	II	A	80
D12	MRD test positivity by qPCR is defined as Ct <40 in ≥2 of 3 replicates.	III	B	73
D13	MRD test negativity by qPCR is defined as Ct ≥40 in at least 2 of 3 replicates, when ≥10 000 copies (optimally, ≥30 000 copies) of the housekeeping gene were measured.	II	A	80
D14	MRD-LL detection using cDNA in *NPM1*-mutated AML is provisionally defined as <2%, but above the detection limit of the assay (ratio of the target and housekeeping genes).[79] MRD-LL is associated with a very low relapse risk in patients with *NPM1* mutations when measured at the end of consolidation chemotherapy.	II	A	67
D15	The optimal NGS-MRD threshold level that best discriminates subsequent relapse risk has not yet been defined for individual mutations, combinations of mutations, or treatment time points. NGS-MRD test positivity (measured on genomic DNA) is provisionally defined as ≥0.1% VAF. Although NGS-MRD test negativity is defined as <0.1% VAF, results <0.1% may still be associated with adverse outcomes and may be reported as molecular MRD-LL.	IV	B	93

Ct, cycling threshold; GoR, grade of recommendation; LoE, level of evidence; LoA, level of agreement. See supplemental Table 6 for definitions of GoR and LoE.

*No Delphi score available. The recommendation was reached after discussions among experts.

Table 4. (continued)

No.	Clinical MRD recommendation	LoE	GoR	LoA (%)
D16	MRD relapse is now defined as either (1) conversion of MRD negativity to MRD positivity, independent of the MRD technique, or (2) increase of MRD ≥1 $\log_{10}$ between any 2 positive samples measured in the same tissue (PB or BM) in patients with MRD-LL.	V	A	86
D17	Conversion from negative to positive MRD in PB or BM should be confirmed within 4 wk, in a second consecutive sample, preferably with a BM sample.	IV	A	89
D18	Available data suggest that patients with 1 positive and 1 negative MRD result from 2 different techniques have a higher relapse risk than patients with 2 negative MRD results, but a lower relapse risk than patients with 2 positive MRD results.	IV	B	95
D19	MRD assay parameters are defined in supplemental Table 4 and should be included in results reports. Scientific reports on MRD studies should include the parameters listed in supplemental Table 5.	V	A	89
D20	Future MRD studies, including clinical trials, should report data using the thresholds and response definitions given in this article.	V	A	94
D21	Failure to achieve MRD^- remission by MFC, molecular MRD positivity after completion of consolidation chemotherapy, and/or MRD relapse (either molecular or MFC, as defined herein) are associated with disease relapse and inferior outcomes. However, select patients with *NPM1* mutations and CBF AML may have prolonged survival, despite MRD-LL (<2%).	III	B	93
D22	For patients who are (1) MRD positive by MFC after 2 cycles of intensive chemotherapy, after consolidation chemotherapy, prior to stem cell transplantation, and/or after stem cell transplantation[83,84]; (2) MRD^+ by ≥2% *NPM1* mutant copies per *ABL1* copies measured in BM or transcript levels of *NPM1* or CBF fusions failed to reach a 3- to 4-log reduction in the same tissue after completion of consolidation chemotherapy (the ratio of target copies/*ABL1* copies between the sample at diagnosis and the sample after completion of consolidation chemotherapy, measured in the same tissue, preferably BM)[37,71,80,85,86]; and/or (3) demonstrated to have MRD relapse (either molecular or MFC), individualized treatment[83] and/or conditioning regimen strategies should be considered, preferably as part of clinical trials, in an effort to reduce disease relapse.	V	C	100
D23	Patients with *NPM1* or CBF AML who have stable molecular MRD-LL do not necessarily require a change in treatment (at end of treatment or during follow-up).	III	B	89
D24	Stable or declining levels of *PML-RARA* by PCR during active treatment of APL should not trigger a change in treatment plan.	V	B	94
D25	Conversion of *PML-RARA* by PCR from undetectable to detectable, and/or a ≥1 $\log_{10}$ increase in high-risk patients with previously stable PML/RARA levels should be regarded as imminent disease relapse in APL, when confirmed in a second sample.	IV	B	88
D26	Pretransplant MRD positivity should not be viewed as a contraindication to stem cell transplantation.	IV	A	100
D27	The panel recommends that patients with detectable MRD before allo-HCT myeloablative conditioning be considered.	II	A	95
D28	All AML clinical trials should monitor molecular and/or MFC-MRD assessments whenever response is assessed in BM.	V	B	100

Ct, cycling threshold; GoR, grade of recommendation; LoE, level of evidence; LoA, level of agreement. See supplemental Table 6 for definitions of GoR and LoE.
*No Delphi score available. The recommendation was reached after discussions among experts.

Molecular MRD testing

Approaches and technical requirements for molecular MRD assessment There are 2 approaches to molecular MRD assessment: polymerase chain reaction (PCR) and next-generation sequencing (NGS).[26] The recommendations are summarized in Table 2. Techniques for molecular MRD assessment should reach a limit of detection of 10^{-3} or lower with technically validated assays,[27] using real-time quantitative PCR (qPCR),

Table 5. Definitions for MRD response categories and MRD relapse

Response category	Abbreviation	Defining criteria
CR with negative MRD	$CR_{MRD}-$	1. Complete morphologic remission and 2. MRD^- in all MRD technologies that were used: a. FC-MRD^- in BM (if MFC-MRD was used). b. qPCR-MRD^- in BM (or in PB after cycle 2 for *NPM1*- and CBF-MRD) (if qPCR-MRD was used). c. NGS-MRD^- in BM (if NGS-MRD was used).
CR with positive MRD	$CR_{MRD}+$	1. Complete morphologic remission, and 2. MFC-MRD^+ in PB and/or BM, or 3. NGS-MRD^+ in PB and/or BM, or 4. qPCR-MRD^+ in PB and/or BM.
CR with molecular MRD detection at low level	CR-MRD-LL	1. Morphologic CR, and 2. Molecular MRD detectable at low level in PB and/or BM (ie, qPCR for *NPM1* <2% or NGS-MRD <0.1%, but above the detection limit of the assay).
MRD relapse	—	1. Conversion of MRD negativity to MRD positivity independent of the MRD technique, or 2. increase in MRD copy numbers ≥1 $\log_{10}$ between any 2 positive samples in patients with CR-MRD-LL who are monitored by qPCR. 3. The result of (1) or (2) should be rapidly confirmed in a second consecutive sample, preferably from the BM.

digital PCR (dPCR), or error-corrected NGS with unique molecular identifiers (recommendation B1).

The recommended PCR approaches include classic qPCR, using fluorescent probes and dPCR. The applicability of PCR is limited to the ~40% to 60% of AML cases with ≥1 targetable abnormalities, including mutated *NPM1*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, *PML-RARA*, *KMT2A-MLLT3*, *DEK-NUP214*, *BCR-ABL*, and *WT1*.[28] Molecular MRD analysis for *NPM1* or fusion genes is usually performed from RNA/cDNA because of the high expression of these genes and thus better sensitivity.[29] Both PB and BM can be used for molecular MRD assessment, though sensitivity may be five- to 10-fold lower in PB than in BM.[30]

Either EDTA or heparin can be used as the anticoagulant on samples for molecular MRD analysis (recommendation B2). A potential inhibitory activity of heparin on PCR reactions has been noted, and the anticoagulant effect should be assessed during assay validation.[31] To avoid hemodilution, only 5 mL of BM aspirate should be used for molecular MRD assessment from the first pull of the syringe (or the first pull after repositioning, if the initial pull is used for MFC-based MRD testing (recommendation B3). BM smears for morphologic assessment (0.2-0.5 mL) should be prepared immediately from a few drops of aspirate from the first pull of the syringe. If PB is used for molecular MRD, ≥10 mL is needed, depending on the white blood cell count and assay characteristics.

The method of cell isolation should be kept consistent, as it may alter the leukemic cell percentage (eg, Ficoll separation of PB to reduce dilution of leukemic cells with normal granulocytes or lysis of whole blood; recommendation B4).

qPCR-based molecular MRD assessment Technical requirements for qPCR are largely unchanged from the 2018 guidelines (supplemental Information).[2] Leukemia-specific PCR assays (eg, for *NPM1*, *PML-RARA*, or *CBF* AML) are preferred over less specific markers, such as *WT1* or *EVI1* expression (recommendation B5). If *WT1* is the only available MRD marker, assessment in PB is preferred because of the higher background levels of *WT1* expression in normal BM.[32]

NGS-based molecular MRD assessment Targeted NGS-based MRD testing using specific mutations identified at diagnosis vs agnostic panel approaches have different strengths and limitations, but both approaches can be considered, depending on sensitivity, turnaround time, resource use, setting (research, clinical trial, clinical routine), and ability to standardize methodology and reporting (recommendation B6).[33]

DNA is the standard nucleic acid used for NGS-MRD testing. Prognostic impact has been shown for selected mutations present at diagnosis and/or in complete remission (CR) samples.[34,35] If a panel approach is used, emerging variants not found at diagnosis should be reported only if confidently detected above background noise (recommendation B7).

For the NGS-MRD assessment, the goal should be a read depth that allows for clear discrimination of the target from noise (supplemental Information). Nucleic acid contamination may be reduced by changing the combinations of multiplex identifiers with target sequences from run to run, and by thorough washing of the sequencer between runs. Diagnostic samples should not be combined with MRD samples in the same run, as highly abundant mutations increase the risk of contamination. Technical requirements for NGS-MRD testing are further detailed in the supplemental Information.

Selection of MRD markers for NGS-MRD Diagnostic AML samples are generally screened for mutations using a multigene panel. For NGS-MRD, we recommend considering all detected mutations as potential MRD markers, with the limitations detailed by recommendations B9 to B11[36] (recommendation B8). This process may apply also to patients with *NPM1* mutations, as *NPM1* mutation–negative relapse has been reported in patients who previously were positive for an *NPM1*

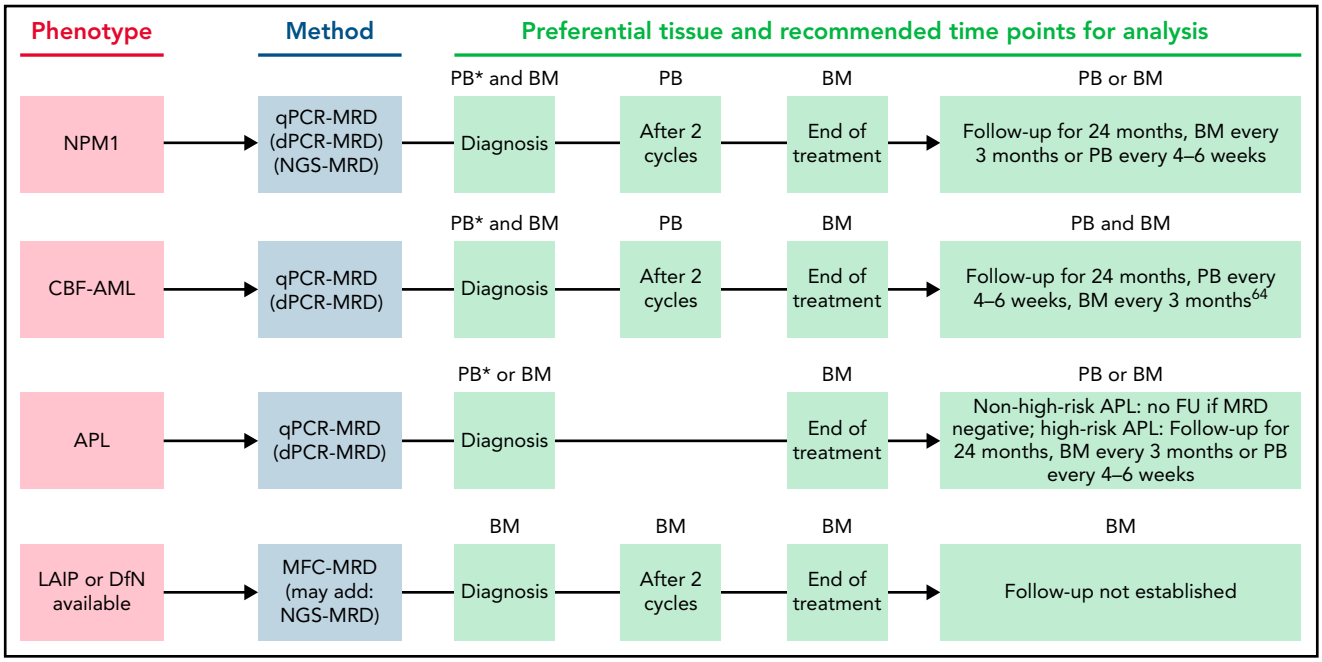

Figure 1. MRD assessment algorithm for different subtypes of AML. *For NPM1 and CBF AML, PB may be used for MRD assessment at diagnosis if there are ≥20% blasts in the PB. If log reduction is used as a measure of MRD response both PB and BM should be analyzed at diagnosis to have both tissues as baseline comparators.

mutation.[37-39] This finding may be especially relevant in patients with morphological or clinical signs of recurrent disease, because AML and MDS developing from clonal hematopoiesis has been documented in *NPM1*$^{-}$ patients during follow-up.[39,40] In addition, of 150 patients with *NPM1* mutations in complete molecular remission, 15% had ≥1 non-DTA (*DNMT3A*, *TET2*, and *ASXL1*) mutation that persisted or was acquired at the time of CR assessment and predicted significantly shorter overall survival.[41]

Germline mutations (variant allele frequency [VAF] of ~50% in genes *ANKRD26*, *CEBPA*, *DDX41*, *ETV6*, *GATA2*, *RUNX1*, and *TP53*) should be excluded as NGS-MRD markers, as they are noninformative for MRD[42] (recommendation B9). DTA mutations can be found in age-related clonal hematopoiesis and should be excluded from MRD analysis (recommendation B10), as mutations associated with clonal hematopoiesis often persist during remission and thus may not represent the leukemic clone.[43-47] If the only detectable mutations are in DTA genes, we recommend using MFC and/or PCR for MRD assessment. Mutations in signaling pathway genes (eg, *FLT3-ITD*, *FLT3-TKD*, *KIT*, *KRAS*, *NRAS*, and others) most likely represent residual AML when detected, but are often subclonal and have a low negative predictive value. These mutations are best used in combination with additional MRD markers (recommendation B11). NGS-MRD analysis in patients treated with targeted agents (FLT3 inhibitors, IDH1/IDH2 inhibitors) should include the molecular marker that is targeted, but also others that are present in the sample (recommendation B12).[48,49] A basic set of genes that covers a large proportion of patients with AML and therefore may be useful in a panel approach is shown in supplemental Table 3.

Bioinformatics analysis for NGS-MRD NGS-MRD data should be interpreted in the context of variant-specific false-positive rates, and laboratory and/or bioinformatics approaches to mitigate sources of error should be used (supplemental Information). As of this writing, there is no uniform bioinformatics pipeline/platform for NGS-MRD variant calling. Harmonization efforts are strongly recommended, preferably using published, open-source algorithms (recommendation B13). Potential cross-sample sequence contamination as a result of pooling samples in NGS-MRD should be bioinformatically evaluated (recommendation B14).

Future goals

General MRD assays, analytical tools, and reporting standards, all require standardization and harmonization. Qualification of each MRD approach is essential for clinical decision making, in particular in light of the planned in vitro diagnostics regulation of the European Union.[50] Interlaboratory tests are being performed within the ELN for MFC, qPCR-based *NPM1* analysis and NGS-MRD, and multicenter initiatives are encouraged.[51] Turnaround time, cost, sensitivity, and effects of clonal evolution should be compared between these approaches. The recommended MRD cutoffs of the major MRD technologies should be validated in the ELN risk groups, and the value of alternative cutoffs should be evaluated. In addition, clinical studies should investigate whether MFC and molecular MRD have distinct applications or should be used in combination for optimal impact.

MFC-MRD testing Further investigation of background levels of aberrant immunophenotypic cell populations in normal and regenerating BM is required to increase assay specificity. Laboratories should gain expertise on background levels by measuring MRD in control samples from different treatment phases with their in-house panels. Also, identification of MFC profiles associated with clonal hematopoiesis[11] could allow for these

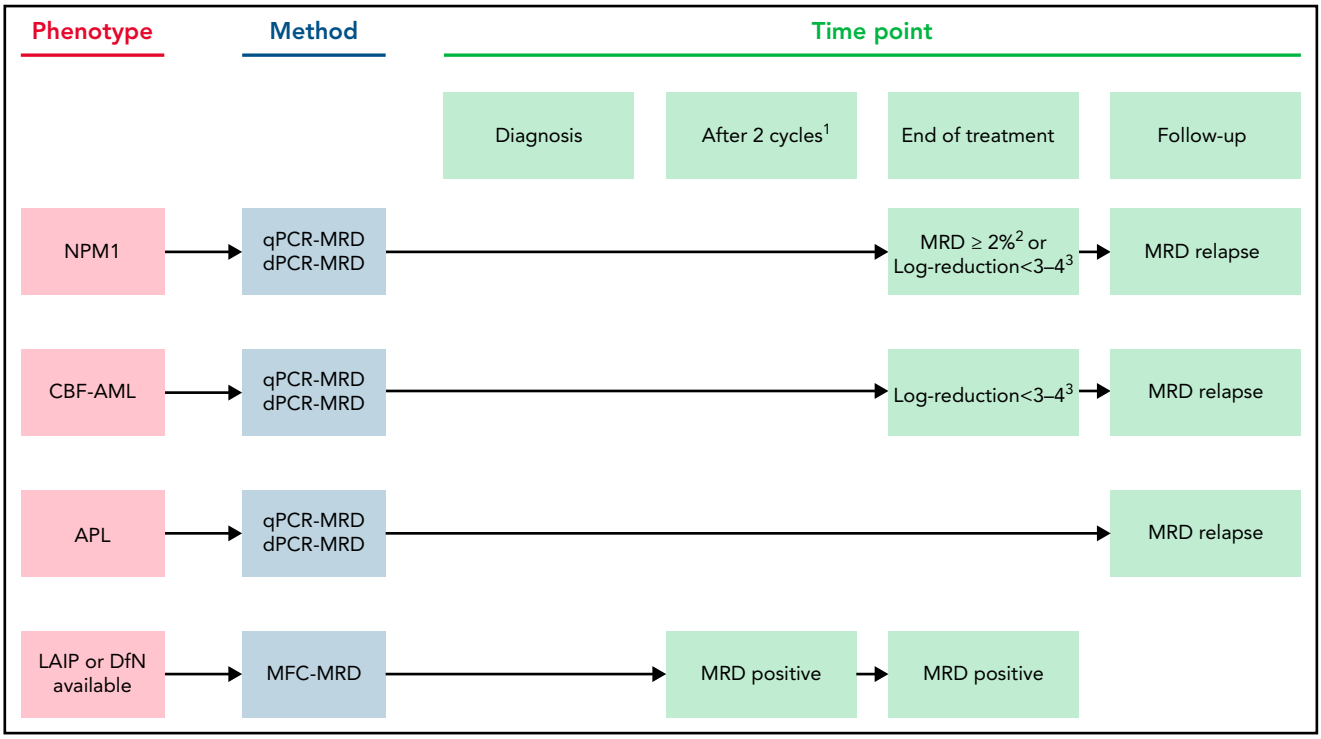

Figure 2. Time points at which MRD is considered a clinically relevant biomarker. The time points and MRD cutoffs are indicated at which an MRD result may influence the therapeutic decision for a given patient. For example, in a patient with AML carrying an *NPM1* mutation, who is monitored by qPCR, MRD persistence at ≥2% *NPM1* mutant copies/*ABL1* copies at the end of chemotherapy may trigger the decision to consider allo-HCT for this patient. *After 2 cycles of chemotherapy (either 2 induction cycles or 1 induction and 1 consolidation cycle), which includes the time point before allo-HCT. **Percentage *NPM1* mutant copies per *ABL1* copies measured in BM. ***Log reduction of the ratio of target copies/*ABL1* copies between the sample at diagnosis and the sample at end of treatment, measured in the same tissue (preferably BM).

populations to be separated from prognostically relevant MFC-MRD populations.[46]

Finally, further evaluation of the role of leukemia stem cells (LSCs)[52-54] for MFC-MRD is recommended (group C recommendations are listed in Table 3). LSCs can be immunophenotypically defined as $CD34^+/CD38^-$ cells[55] combined with an aberrant marker not present on hematopoietic stem cells (HSCs) (eg, CD45RA [PTPRC], CLL-1 [CLEC12A], or CD123 [IL3RA]) (recommendation C1). Measurements of LSCs may have prognostic value and should be further validated in prospective clinical trials (recommendation C2). LSC detection requires optimally 4 million events, which is most likely best achieved with a 1-tube assay (recommendation C3).[56]

Gating of relevant cell populations is still considered subjective, time consuming, and requires expertise. Therefore, automated flow analyses are currently being explored.[4,57-59] High-quality flow cytometry data (standardized instrument settings, preanalytics, and measurements) are necessary for future automated analyses (recommendation C4).

Molecular MRD testing For qPCR-MRD, the prognostic value of log reduction of transcript levels between diagnosis and post-induction time points is under evaluation in clinical trials. For NGS-MRD, the prognostic and predictive relevance of different time points, tissues, and target genes are all under investigation. Bioinformatics approaches also need standardization and quality control rounds. Further studies are needed on how to interpret NGS results when monitoring several gene mutations in a single patient, and whether there are prognostic differences if one, some, or all genes remain detectable. Finally, it is important to identify the benefits and limitations of targeted vs panel approaches for NGS-MRD assessment.[60]

Clinical implementation

MRD assessment in AML can be used as (1) a prognostic/predictive biomarker to refine risk assessment and inform treatment decision-making, (2) a monitoring tool to identify impending relapse, and (3) a potential surrogate end point for overall survival in clinical trials to accelerate the development of novel treatment strategies (clinical MRD recommendations are listed in Table 4).

MRD as a prognostic risk factor

MRD should be assessed to refine relapse risk in patients who achieve morphologic remission, with full or partial hematologic recovery ($CR/CR_i/CR_p/CR_h$)[1] (recommendation D1). MRD positivity in AML patients treated with intensive chemotherapy is associated with inferior outcomes.[1] Preliminary data suggest that MRD positivity after nonintensive induction is also associated with poor outcomes.[61-64]

Selecting the technique, material, and appropriate time points for MRD assessment

MFC-MRD has been established as a prognostic factor after induction chemotherapy on BM.[65-68] Particularly for longer-

term follow-up, MRD monitoring using PB would be beneficial and may be informative from recent evidence; however, further research is needed with regard to its sensitivity and specificity.[57,59,69,70]

Ideally, potential MRD markers should be identified at diagnosis using MFC and molecular techniques. If no diagnostic material is available for comparison, MRD can be assessed by using MFC or NGS with the DfN approach or an agnostic gene panel. To confirm remission, MRD assessment should be performed routinely on all BM specimens obtained. Except in the specific molecular subgroups below, MRD monitoring using PB is investigational.

For patients with mutant *NPM1*, CBF AML (*RUNX1-RUNX1T1* or *CBFB-MYH11*), or acute promyelocytic leukemia (APL) (*PML-RARA*), we recommend molecular MRD assessment by qPCR or dPCR (recommendation D2). Patients with AML outside these molecularly defined subgroups should be monitored for MRD by MFC (recommendation D3). NGS-MRD monitoring is useful to refine prognosis in addition to MFC but, to date, there are insufficient data to recommend NGS-MRD as a stand-alone technique (recommendation D4).[44]

In *NPM1*-mutated AML, MRD should be assessed preferentially in PB after 2 cycles of chemotherapy, in BM at the end of consolidation, and in BM every 3 months for 24 months after the end of consolidation. Alternatively, MRD may be assessed from PB every 4 to 6 weeks during follow-up for 24 months (recommendation D5).

In *RUNX1-RUNX1T1*– and *CBFB-MYH11*–mutated AML MRD should be assessed preferentially in PB after 2 cycles of chemotherapy, in BM at the end of consolidation treatment, and in PB every 4 to 6 weeks for 24 months after the end of consolidation (recommendation D6).[71,72]In APL, the most important MRD end point is PCR negativity for *PML-RARA* at the end of consolidation (recommendation D7). For patients with non–high-risk APL, MRD monitoring is only recommended after completion of consolidation and may be discontinued once BM MRD-negativity is achieved (recommendation D8). For high-risk APL, MRD should be assessed by qPCR from BM every 3 months for 24 months, starting at the end of treatment. Alternatively, MRD may be assessed from PB every 4 to 6 weeks during follow-up (recommendation D8a, no Delphi score available).[73-75] Based on the relapse kinetics of patients with high-risk APL treated with ATRA-based regimens, monitoring for 24 months appears sufficient.[75,76]

Ongoing molecular MRD monitoring beyond 24 months of follow-up should be based on individual clinical features (recommendation D9).

Patients who are observed by using MFC-MRD should have BM assessment after 2 cycles of chemotherapy, at the end of consolidation, and before stem cell transplantation, if applicable (recommendation D10).[4] The clinical utility of serial NGS MRD is uncertain, but can be considered by examining BM or PB after 2 cycles of intensive chemotherapy, before stem cell transplantation, at the end of treatment, and during follow-up.[30,35,36,44,77]

MRD response and relapse

MRD thresholds MFC-MRD test positivity is defined as ≥0.1% of CD45-expressing cells with the target immunophenotype (recommendation D11). This threshold guarantees that LAIP sensitivity in normal or regenerating BM is above the frequency of any possible background[12] and is consistent with guidance from the US Food and Drug Administration that the assay be technically validated 1-log below the chosen threshold for clinical decision making.[24] However, data from clinical trials suggest that MRD levels below 0.1% may still indicate active disease. For example, a positivity threshold of 0.035% has been prospectively validated in the context of a clinical trial (GIMEMA AML1310),[6] and other studies have also demonstrated prognostic relevance when using "any detectable MRD" as a threshold for MRD positivity.[67,68,78]

MRD test positivity by qPCR is defined as a cycling threshold <40 in ≥2 of 3 replicates (recommendation D12). MRD test negativity by qPCR is defined as a cycling threshold ≥40 in ≥2 of 3 replicates, when at least 10 000 copies (but optimally, ≥30 000 copies) of the housekeeping gene *ABL1* (or comparable numbers for other housekeeping genes, eg, *GUS* and *B2M*) were measured (recommendation D13). Low-level molecular MRD detection using cDNA in *NPM1* mutated AML (MRD at low level [MRD-LL], previously called molecular persistence with low copy numbers) is provisionally defined as <2% but above the detection limit of the assay (ratio of the target and housekeeping genes).[79] MRD-LL is associated with a very low relapse risk in patients with *NPM1* mutations when measured at the end of consolidation chemotherapy (recommendation D14). The optimal dPCR threshold level has not yet been evaluated in sufficiently large patient cohorts. dPCR test positivity (measured on genomic DNA) is provisionally defined as ≥0.2% VAF. The discriminating threshold for dPCR when using complementary DNA needs further validation.

The optimal NGS-MRD threshold level that best discriminates subsequent relapse risk has not yet been defined for individual mutations, combinations of mutations, or treatment time points. NGS-MRD test positivity (measured on genomic DNA) is provisionally defined as ≥0.1% VAF. Though NGS-MRD test negativity is defined as <0.1% VAF, results <0.1% may still be associated with adverse outcomes and may be reported as molecular MRD-LL (recommendation D15).

Definition of MRD response and MRD relapse MRD relapse (Table 5) is now defined as either (1) conversion of MRD negativity to MRD positivity independent of the MRD technique or (2) increase of MRD ≥1 $\log_{10}$ between any 2 positive samples measured in the same tissue (PB or BM) in patients with MRD-LL (recommendation D16). Conversion from negative to positive MRD in PB or BM should be confirmed within 4 weeks, in a second consecutive sample, preferably with a BM sample (recommendation D17).

Integration of multimodality MRD results

MRD positivity by any methodology is sufficient to suspect poor clinical risk. Available data suggest that patients with 1 positive and 1 negative MRD result from 2 different techniques have a higher relapse risk than patients with 2 negative MRD results, but a lower relapse risk than patients with 2 positive MRD

results[43,44] (recommendation D18). Future studies are needed to integrate the results of multiple MRD assays into 1 prognostic score.

How to report MRD results

MRD assay parameters are defined in supplemental Table 4 and should be included in results reports. Scientific reports on MRD studies should include the parameters listed in supplemental Table 5 (recommendation D19). Future MRD studies, including clinical trials, should report data according to the thresholds and response definitions used herein (recommendation D20).

Clinical consequences of MRD assessment

Failure to achieve MRD$^-$ remission by MFC, molecular MRD positivity after completion of consolidation chemotherapy, and/or MRD relapse (either molecular or MFC) are associated with disease relapse and inferior outcomes. However, select patients with *NPM1* mutations and CBF AML may have prolonged survival despite low-level molecular MRD (<2%, MRD-LL)[80-82] (recommendation D21).

For patients who are (1) MRD$^+$ by MFC after 2 cycles of intensive chemotherapy, after consolidation chemotherapy, prior to stem cell transplantation, and/or after stem cell transplantation[83,84]; (2) MRD$^+$ by ≥2% *NPM1* mutant copies per *ABL1* copy measured in BM or transcript levels of *NPM1* or core binding factor (CBF) fusions failed to reach a 3- to 4-log reduction in the same tissue after completion of consolidation chemotherapy (ratio of target copies/*ABL1* copies between the sample at diagnosis and the sample after completion of consolidation chemotherapy, measured in the same tissue, preferably BM)[37,71,80,85,86]; and/or (3) demonstrated to have MRD relapse (either molecular or MFC), individualized treatment[83] and/or conditioning regimen strategies should be considered, preferably as part of clinical trials, in an effort to reduce disease relapse (recommendation D22; Figure 1). However, it should be emphasized that a single positive MRD test does not guarantee relapse and should not be used as the sole basis for clinical action.

Patients with *NPM1* or CBF AML who have stable molecular MRD-LL do not necessarily require a change in treatment, at the end of treatment or during follow-up[80] (recommendation D23).

Stable or declining levels of *PML-RARA* by PCR during active treatment of APL should not trigger a change in treatment plan (recommendation D24). Conversion of *PML-RARA* by PCR from undetectable to detectable, and/or a ≥1log$_{10}$ increase in high-risk patients with previously stable PML-RARA levels should be regarded as imminent disease relapse in APL, when confirmed in a repeat sample (recommendation D25; Figure 2).

In ELN intermediate-risk patients, MRD negativity in BM measured by MFC after 2 cycles of chemotherapy justifies consideration of consolidation chemotherapy or autologous stem cell transplantation as potential alternatives to allogeneic hematopoietic cell transplantation (allo-HCT) for eligible patients.[6,7] All eligible ELN adverse risk patients should undergo allo-HCT, regardless of MRD. MRD positivity and/or MRD relapse at the end of treatment, during maintenance and follow-up are associated with poor outcome and justify consideration of salvage treatment options, including allo-HCT.[30,84,87,88]

Pretransplant MRD positivity should not be viewed as a contraindication to stem cell transplantation (recommendation D26).[89] The panel recommends that patients with detectable MRD before allo-HCT be considered for myeloablative conditioning (recommendation D27), noting that other approaches, such as post–allo-HCT maintenance treatment or donor lymphocyte infusions, may also reduce relapse risk.[35,49,90-92]

Use of MRD as a surrogate end point for drug testing

The strong negative prognostic impact of MRD positivity in AML has sparked interest in using MRD as a surrogate efficacy-response biomarker to accelerate drug development/testing and regulatory approval.[26] The US Food and Drug Administration has issued a guidance document on the regulatory considerations for the use of MRD in clinical trials.[24] Important factors for establishing surrogacy are biological plausibility, results from epidemiological studies demonstrating the prognostic value of the surrogate end point (eg, achieving an MRD$^-$ remission must correlate with longer survival than achieving an MRD$^+$ remission), and evidence from clinical trials showing that treatment effects on the surrogate end point correspond to treatment effects on the clinical outcome (ie, an experimental treatment must increase both MRD$^-$ remissions and survival, compared with the control treatment). Currently, although some data from mostly nonrandomized trials show a treatment effect on both MRD responses and survival,[93-96] robust data from randomized trials are limited.[80,97] Therefore, all AML clinical trials should monitor molecular and/or MFC-MRD assessments whenever response is assessed in BM (recommendation D28).[61]

Suggestion for further improvements in clinical implementation

Future studies should evaluate whether MRD assessment is feasible and has prognostic value in patients who achieve a morphologic leukemia-free state. The prognostic relevance of MRD in nonintensive AML treatment regimens[67] should be further assessed. Also, the relevance and prognostic value of MRD in first salvage and beyond have not been established and should be further investigated. Finally, it is of critical importance to prospectively assess the outcomes of MRD-directed interventions (eg, dose reductions or treatment interruptions in MRD$^-$ patients, or treatment intensification or modification in patients with detectable MRD).

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Authorship

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Footnotes

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