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# 2021 Update Measurable Residual Disease in Acute Myeloid Leukemia: European LeukemiaNet Working Party Consensus Document

Tracking no: BLD-2021-013626R1

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**Abstract:**
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 evaluates 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 two-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 (NGS)-based MRD testing and integrative assessments of MRD irrespective of technology. Other topics include use of MRD as a prognostic and surrogate endpoint 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.

**Conflict of interest:** COI declared - see note

**COI notes:** AAL: Advisory role for Celgene/BMS, Amgen, Novartis, Takeda; Research funding to institute from Alexion. AM: Nothing to disclose AV: Advisory role for Novartis, Pfizer, Jazz Pharmaceuticals, Amgen, Abbvie, Gilead, Astellas, Incyte, Janssen & Cylag; Research funding to the Department of Biomedicine and Prevention, University Tor Vergata from Sandoz and Jazz Pharmaceuticals; Speaker bureau for Pfizer AW: Advisory role for Amgen, Pfizer, Abbvie, BMS, Astellas, Janssen; Honoria from Abbvie, Novartis, Roche, Astellas, and Janssen. BD: Nothing to disclose BR: Nothing to disclose JC: Advisory role for Novartis; Research grant for institution Novartis, Merus, Takeda, Genentech CB: Nothing to disclose CBAER: Employment by MLL, Munich Leukemia Laboratory CP: Personal fees and travel/accommodations from Amgen, Janssen, Novartis, Bristol Meyer Scribb, Abbvie, Astellas; Grants and personal fees and travel/accommodations from Celgene; Grants from Merck; Travel and hotel accommodation

from Innate pharma, Ariad, Daiichi Sakyo. CSH: Research support for institution: Sellas. CT: Advisory role for JAZZ, Novartis; Honoraria from JAZZ, Janssen, Novartis, Astellas, Illumina, Thermo Scientific; Research funding to institution from Novartis, JAZZ; Ownership: AgenDix. FB: Advisory role for Novartis; Speakers bureau for Novartis. FR: To be completed FT: Advisory role for Celgene/BMS, Novartis, Abbvie Daichi, Pfizer; Research Support for Institution: Celgene/BMS, Novartis. GO: Advisory role for Novartis, Pfizer, BMS, Janssen, Celgene, AGIOS, Amgen, Gilead, Astellas, Roche, Jazz Pharmaceuticals, Merus; Consultancy for Janssen, Celgene, Roche; Research support to institute from Novartis, Janssen, Celgene. JP: Nothing to disclose JT: Nothing to disclose KD: Advisory role for Abbvie, BMS/Celgene, Daiichi Sankyo, Jazz Pharmaceuticals, Janssen, Novartis, Roche, Honoraria from BMS/Celgene, Daiichi Sankyo, Jazz Pharmaceuticals, Janssen, Novartis, Roche; Research funding to institution from Astellas, Agios, Novartis. LLN: Nothing to disclose LM: Advisory role for Abbvie, Janssen, Novartis and BMS/Celgene. MH: Advisory role for Abbvie, BMS/Celgene, Daiichi Sankyo, Jazz Pharmaceuticals, Novartis, Pfizer, Roche, Tolremo; Honoraria from Jazz Pharmaceuticals, Janssen, Novartis; Research funding to institution from Astellas, Bayer Pharma AG, BergenBio, Daiichi Sankyo, Jazz Pharmaceuticals, Karyopharm, Novartis, Pfizer, Roche. MLG: Advisory role for BridgeMedicines, SeqRx, Epsilen Bio. Research funding from Cellectis, BridgeMedicines, Daiichi Sankyo. MS: Advisory role for Amgen, Celgene, Gilead, Janssen, Novartis, Pfizer, and Seattle Genetics; Research funding to institution from Amgen, Gilead, Miltenyi Biotec, Morphosys, Roche, and Seattle Genetics; Consultancy for Amgen, BMS, Celgene, Gilead, Pfizer, Novartis, and Roche; Speakers' bureau at Amgen, Celgene, Gilead, Janssen, and Pfizer. PV: Noting to disclose VB: Advisory role for Amgen, Gilead, and Pfizer; Research funding to institution from Gilead, Celgene, and Novartis. RD: Advisory role for Abbvie, Jazz Pharmacueticals, Menarini, Novartis, Pfizer; Research support to institution from Abbvie, Amgen; Consultancy for Abbvie, Astellas, Jazz Pharmaceuticals, Pfizer; Speaker bureau for Astellas, Jazz Pharmaceuticals, Novartis, Pfizer. SDF: Speaker bureau for Pfizer, Jazz. TH: Part owner of MLL, Munich Leukemia Laboratory VB: Nothing to disclose WK: Part owner of MLL, Munich Leukemia Laboratory YO: Advisory role for Amgen, Pfizer, Abbvie, BMS, Astellas, Janssen; Honoria from Abbvie, Novartis, Roche, Astellas, and Janssen.

**Preprint server**: No;

**Author contributions and disclosures**: Conception and design of the manuscript: MH, JC Organization and analysis of Delphi polls and meetings: JC, MH, LLN, JT Participation in Delphi polls and group discussions: All authors Writing of manuscript drafts: JC, MH Revision and final acceptance of manuscript: All authors

**Non-author contributions and disclosures**: No;

**Agreement to Share Publication-Related Data and Data Sharing Statement**:

**Clinical trial registration information (if any)**:

# 2021 Update on Measurable Residual Disease (MRD) in Acute Myeloid Leukemia (AML): A Consensus Document from the European LeukemiaNet MRD Working Party

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**Key Points**

1. The updated ELN Working Party guidelines account for the rapid development in the field of MRD testing for AML.
2. All recommendations were graded by level of evidence, grade of recommendation and level of agreement based on a two-stage Delphi poll.

**Word count**

Abstract: 196; Main Text: 4,448; 2 Tables: 2; 2 Figures; 1 Supplementary information file including 5 supplementary Tables.

**Abstract**

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 evaluates 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 two-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 (NGS)-based MRD testing and integrative assessments of MRD irrespective of technology. Other topics include use of MRD as a prognostic and surrogate endpoint 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 AML patients.[1] MRD monitoring may have value in guiding post-remission therapy and identifying early relapse and as a surrogate endpoint 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.

The goal of the 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 using a Delphi poll (methods in Supplemental information and Table S1) and the overall results are summarized in Tables 1a-d.[3]

### *Terminology*

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 non-zero result may still be called “negative” by a laboratory if the level detected is below the threshold linked to prognosis.

## I TECHNOLOGIES

### 1. Multiparametric Flow Cytometry (MFC) MRD Testing

Immunophenotyping is an essential, readily available tool for diagnosing AML and is currently the most commonly used MRD detection methodology. Supplemental Table S2 summarizes recent clinical studies incorporating MFC-MRD assessment in AML, including for randomized treatment comparisons[4,5] and MRD-directed therapy.[6,7] Here, we update current best practices (Table 1a). Our consensus recommendations for optimized technical requirements for MFC MRD are described in a separate manuscript (Tettero et al., submitted).

*a) "Leukemia-Associated ImmunoPhenotype" (LAIP) and "Different from Normal" (DfN)*

The flow cytometry expert panel continues to recommend integration of diagnostic LAIP and DfN aberrant immunophenotype approaches to allow 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 if a patient has diagnostic flow cytometric MRD targets that can be tracked **(recommendation A1)**. Implementation of a common, minimum required set of tubes/fluorochromes is a prerequisite for harmonized MRD 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, 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+CD38low/- compartment, when markers such as CD56, CD7 and others like CD45RA designated as leukemic stem cell markers are aberrantly expressed. In cases with a monocytic component, additional markers (e.g., CD64, CD11b, CD4) may also be relevant.[8] The DfN approach detects aberrant clones regardless of immunophenotypic shifts, since 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).[9] The panel advises the combined LAIP/DfN approach, but notes that some abnormal immunophenotypes may appear and/or disappear during monitoring, potentially due to transient expression on regenerating non-leukemic progenitors.[10–12] This phenomenon may affect the respective specificities of both LAIP and DfN MRD detection, in particular when the percentages of LAIPs at lower thresholds (e.g. <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 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 weeks, if clinically indicated, may be informative **(recommendation A5)**.

*b) Sampling and pre-analytical 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.[13] 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 two accepted techniques: 1) bulk lysis, followed by wash/stain/wash; or 2) stain/lyse/wash or no-wash.[9,14] Whichever technique is selected should reliably produce high quality MFC measurements (i.e. optimal cell concentration and no loss of forward or sideward scatter properties) and should be applied consistently across samples.

Basic principles for instrument settings are described elsewhere and we suggest using standard operating procedures developed by international flow cytometry consortia.[15,16] Also, efforts should be made to evaluate sample quality with respect to PB contamination.[17,18] 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)**.

*c) 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)**.[19–22] Acquisition should collect the highest possible number of relevant events and, accordingly, to ensure quality of relevant events acquisition, use a gating syntax including Forward Scatter (FSC) versus time and doublet exclusion plots [e.g. 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 Side Scatter (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(s) available **(recommendation A11)**.

In order 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 negative results without LLOD information is not meaningful.[23] 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.[24]

## 2. Molecular MRD Testing

*a) Approaches and technical requirements for molecular MRD assessment*

There are two approaches to molecular MRD assessment: PCR and next generation sequencing (NGS).[25] The recommendations are summarized in Table 1b. Techniques for molecular MRD assessment should reach a limit of detection (LOD) of $10^{-3}$ or lower with technically validated assays[26] using quantitative polymerase chain reaction (qPCR), digital PCR (dPCR), or error corrected NGS with unique molecular identifiers (UMIs) **(recommendation B1**).

The recommended PCR approaches include classical quantitative real time PCR (qPCR) using fluorescent probes and digital PCR (dPCR). The applicability of PCR is limited to the approximately 40-60% of AML cases with one or more targetable abnormalities, including mutated *NPM1*, *RUNX1-RUNX1T1, CBFB-MYH11, PML-RARA, KMT2A-MLLT3, DEK-NUP214, BCR-ABL* and *WT1*.[27] Molecular MRD analysis for *NPM1* or fusion genes is usually performed from RNA/cDNA due to the high expression of these genes and thus better sensitivity.[28] Both PB and BM can be used for molecular MRD assessment, though sensitivity may be 5-10 fold lower in PB compared to BM.[29]

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.[30]

To avoid hemodilution, only 5 ml of bone marrow 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 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 syringe. If PB is used for molecular MRD, at least 10 mL is required, 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 (e.g., Ficoll separation of PB to reduce dilution of leukemic cells with normal granulocytes or lysis of whole blood; **recommendation B4)**.

*b) qPCR-based molecular MRD assessment*

Technical requirements for qPCR are largely unchanged from the 2018 guidelines (see Supplemental Information).[2] Leukemia-specific PCR assays (e.g. for *NPM1, PML-RARA*, or *CBF* AML) are preferred over less specific markers like *WT1* or *EVI1* expression

**(recommendation B5)**. If *WT1* is the only available MRD marker, assessment in PB is preferred due to higher background levels of *WT1* expression in normal BM.[31]

*c) NGS-based molecular MRD assessment*

Targeted NGS-based MRD testing using specific mutations identified at diagnosis versus 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)**.[32]

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 CR samples.[33,34] 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 clear discrimination of the target from noise (see 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 Supplement.

*d) Selection of MRD markers for NGS-MRD*

Diagnostic AML samples are generally screened for mutations using a multi-gene panel. For NGS-MRD, we recommend considering all detected mutations as potential MRD markers, with the limitations detailed below[35] **(recommendation B8)**. This may apply also to *NPM1* mutated patients, as *NPM1* mutation negative relapse was reported in patients who previously were *NPM1* mutation positive[36–38]. This might be especially relevant in patients with morphological or clinical signs of recurrent disease, since AML and MDS developing from clonal hematopoiesis has been documented in *NPM1*-negative patients during follow-up[38,39]. In addition, of 150 *NPM1* mutated patients in complete molecular remission, 15% had at least one non-DTA (*DNMT3A, TET2, ASXL1*) mutation that persisted or was acquired at the time of CR assessment and predicted significantly shorter overall survival[40].

Germline mutations (VAF of ~ 50% in genes *ANKRD26, CEBPA, DDX41, ETV6, GATA2, RUNX1, TP53*) should be excluded as NGS-MRD markers, as they are non-informative for MRD[41] **(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.[42–46] If the only detectable mutations are in DTA genes, we recommend using MFC and/or PCR for MRD assessment. Mutations in signaling pathway genes (e.g., *FLT3-ITD, FLT3-TKD, KIT, KRAS, NRAS* etc.) 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)**.[47,48] A basic set of genes that covers a large proportion of AML patients and therefore may be useful in a panel approach is shown in Supplemental Table S3.

*e) 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 employed (see 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)**.

### *3. 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 (IVDR) of the European Union.[49] Inter-laboratory tests are being performed within the ELN for MFC, qPCR-based *NPM1* analysis and NGS-MRD, and multicenter initiatives are encouraged.[50] 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 cut-points 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[10] could allow these populations to be separated from prognostically relevant MFC-MRD populations.[45]

Finally, further evaluation of the role of leukemia stem cells (LSCs)[51–53] for MFC-MRD is recommended (Table 1c). LSCs can be immunophenotypically defined as CD34+/CD38- cells[54] combined with an aberrant marker not present on HSCs, e.g. 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, likely best achieved with a one tube assay **(recommendation C3)**.[55]

Gating of relevant cell populations is still considered subjective, time consuming and requiring expertise. Therefore, automated flow analyses are currently being explored.[4,56–58] High quality flow cytometry data (standardized instrument settings, pre-analytics and measurements) are required 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 required 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.[59]

## II CLINICAL IMPLEMENTATION

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

### 1. MRD as 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 non-intensive induction is also associated with poor outcomes.[60–63]

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

MFC MRD has been established as prognostic factor after induction chemotherapy on BM.[64–67] 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.[56,68–70]

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 using MFC or NGS with the DfN approach or an agnostic gene panel, respectively. MRD assessment should be performed routinely on all bone marrow specimens obtained to confirm remission. 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 APL (*PML-RARA*), we recommend molecular MRD assessment by qPCR or dPCR **(recommendation D2)**. AML patients outside these molecularly defined subgroups should be monitored for MRD using 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)**.[43]

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 4-6 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 end of consolidation treatment,

and in PB every 4-6 weeks for 24 months after the end of consolidation **(recommendation D6)**.[71,72]

In APL, the most important MRD endpoint is PCR negativity for *PML-RARA* at the end of consolidation (recommendation D7). For non-high-risk APL patients, 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 4-6 weeks during follow up **(recommendation D8a,** no Delphi score available**)**.[73–75] Based on the relapse kinetics of high-risk APL patients treated with ATRA-based regimens, monitoring for 24 months appears sufficient.[75–77]

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

Patients who are followed using MFC-MRD should have BM assessment after 2 cycles of chemotherapy, at the end of consolidation, and prior to stem cell transplantation, if applicable **(recommendation D10)**.[4] The clinical utility of serial NGS MRD is uncertain, but can be considered using BM or PB after two cycles of intensive chemotherapy, prior to stem cell transplantation, at end of treatment, and during follow-up.[29,34,35,43,78]

### 3. MRD response and relapse

*a) 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[11] and is consistent with guidance from the Food and Drug Administration that the assay be technically validated 1-log below the chosen threshold for clinical decision-making.[23] 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 threshold for MRD positivity.[66,67,79]

MRD test positivity by qPCR is defined as cycling threshold (Ct) <40 in at least 2 of 3 replicates (recommendation D12). MRD test negativity by qPCR is defined as Ct ≥40 in at least 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, e.g. *GUS*, *ß2M*) 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 MP-LCN) is provisionally defined as <2% but above the detection limit of the assay (ratio of the target and housekeeping genes)[80]. MRD-LL is associated with a very low relapse risk in *NPM1*-mutated patients 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 cDNA 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 detection at low level **(MRD-LL, recommendation D15)**.

*b) Definition of MRD response and MRD relapse (Table 2)*

MRD relapse is now defined as either a) conversion of MRD negativity to MRD positivity independent of the MRD technique or b) increase of MRD ≥ 1 $\log_{10}$ between any two 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)**.

## 4. Integration of multi-modality MRD results

MRD positivity by any methodology is sufficient to suspect poor clinical risk. Available data suggest that patients with one positive and one negative MRD result from two different techniques have a higher relapse risk than patients with two negative MRD results, but a lower relapse risk than patients with two positive MRD results[42,43] **(recommendation D18)**. Future studies are needed to integrate the results of multiple MRD assays into one prognostic score.

## 5. How to report MRD results

MRD assay parameters are defined in Supplemental Table S4 and should be included in results reports. Scientific reports on MRD studies should include the parameters listed in Supplemental Table S5 **(recommendation D19)**. Future MRD studies, including clinical

trials, should report data using the thresholds and response definitions in this manuscript **(recommendation D20)**.

## 6. Clinical consequences of MRD assessment

Failure to achieve MRD-negative remission by MFC, molecular MRD-positivity after completion of consolidation chemotherapy, and/or MRD relapse (either molecular or MFC, as defined above) are associated with disease relapse and inferior outcomes. However, selected patients with *NPM1* mutations and CBF AML may have prolonged survival despite low-level molecular MRD (<2%, MRD-LL)[81–83] **(recommendation D21)**.

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;[84,85] (2) MRD positive by ≥2% *NPM1* mutant copies per *ABL1* copies measured in BM or transcript levels of *NPM1* or CBF fusions failed to reach a 3-4 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);[36,71,81,86,87] and/or (3) demonstrated to have MRD relapse (either molecular or MFC), individualized treatment[84] 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 detection at low level (MRD-LL) do NOT necessarily require a change in treatment (at EOT or during follow up)[81] **(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 ≥ 1 log10 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 two cycles of chemotherapy justifies consideration of consolidation chemotherapy or autologous stem cell transplantation as potential alternatives to alloHCT for eligible patients.[6,7] All eligible ELN adverse risk patients should undergo alloHCT, 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 alloHCT.[29,85,88,89]

Pre-transplant MRD positivity should not be viewed as a contraindication to stem cell transplantation **(recommendation D26)**.[90] The panel recommends that patients with detectable MRD before alloHCT should be considered for myeloablative conditioning **(recommendation D27)** noting that other approaches such as post-alloHCT maintenance treatment or donor lymphocyte infusions may also reduce relapse risk.[34,48,91–93]

**7. 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.[25] The U.S. Food & Drug Administration (FDA) has issued a guidance document on the regulatory considerations for the use of MRD in clinical trials.[23] Important factors for establishing surrogacy are biological plausibility, results from epidemiological studies demonstrating the prognostic value of the surrogate end point (e.g. achieving a MRD-negative remission must correlate with longer survival than achieving a MRD-positive remission), and evidence from clinical trials showing that treatment effects on the surrogate endpoint correspond to treatment effects on the clinical outcome (i.e. an experimental treatment needs to increase both MRD-negative remissions and survival, compared to the control treatment. Currently, while some data from mostly non-randomized trials show a treatment effect on both MRD responses and survival,[94–97] robust data from randomized trials are limited.[81,98] Therefore, all AML clinical trials should monitor molecular and/or MFC MRD assessments whenever response is assessed in BM **(recommendation D28)**.[60]

**8. 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 (MLFS). The prognostic relevance of MRD in non-intensive AML treatment regimens[66] 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, e.g. dose reductions or treatment interruptions in MRD negative patients, or treatment intensification or modification in patients with detectable MRD.

**Acknowledgments**

The authors would like to thank Ruediger Hehlmann for his continuous generous support of the ELN AML MRD Working Party on behalf of the European LeukemiaNet. This work was supported in part by the Intramural Research Program of the National Heart, Lung, and Blood Institute.

**Authorship Contributions**

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**Conflict of Interest Disclosures**

The disclosures of all authors can be found in Supplemental Information.

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## Tables

**Table 1a. 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 a) determine if a patient has a diagnostic flow cytometric MRD target, b) 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 core MRD markers CD34, CD117, CD45, CD33, CD13, CD56, CD7, HLA-DR to assess all samples. | V | B | 88% |
| A4 | Particular attention should be devoted to evaluating 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 | V | C | 94% |

| No. | Multiparameter Flow Cytometry MRD recommendation | LoE | GoR | LoA |
|---|---|---|---|---|
| | possibility and note that a repeat sample in 2-4 weeks, if clinically indicated, may be informative. | | | |
| **A6** | Request first pull BM aspirate for MRD, process sample within 3 days of storage, undiluted, at ambient conditions. | V | A | 94% |
| **A7** | For samples stored at ambient temperature >3 days, 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 versus 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 a least 100 viable cells in the blast compartment assessed for the best aberrancy(s) available. | V | B | 76% |
| **A12** | LLOD and LLOQ should be calculated to assess MFC-MRD assay performance. | V | B | 93% |

**Table 1b. 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 a LOD of $10^{-3}$ or lower. To achieve this LOD, qPCR, dPCR or error corrected NGS using unique molecular identifiers (UMIs) are recommended. | IV | B | 100% |
| **B2** | Either EDTA or heparin can be used as anticoagulant on samples for molecular MRD analysis. | V | C | 76% |
| **B3** | Only 5 ml of bone marrow 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 (e.g. Ficoll separation to reduce dilution of leukemic cells with normal granulocytes or lysis of whole blood). | V | B | 82% |
| **B5** | Leukemia-specific PCR assays (e.g. for *NPM1, PML-RARA*, or *CBF* AML) are preferred over less specific markers like *WT1* or *EVI1* expression. | V | B | 78% |
| **B6** | Targeted NGS-MRD using specific mutations identified at diagnosis versus 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. | 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 9-11. | IV | B | 100% |
| **B9** | Germline mutations (VAF of ~ 50% in genes *ANKRD26, CEBPA, DDX41, ETV6, GATA2, RUNX1, TP53*) should be excluded as NGS-MRD markers, as they are non-informative for MRD. | V | A | 94% |

| No. | Molecular MRD recommendation | LoE | GoR | LoA |
|---|---|---|---|---|
| **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 (e.g. *FLT3-ITD, FLT3-TKD, KIT, RAS* etc.) 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, 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% |

**Table 1c. 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[54] combined with an aberrant marker not present on HSCs, e.g. 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, likely best achieved with a one tube assay. | V | B | 78% |
| C4 | High quality flow cytometry data (standardized instrument settings, pre-analytics and measurements) are required for future automated analyses. | IV | A | 100% |

**Table 1d. 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 not included in the molecularly defined subgroups above 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 months for 24 months after the end of consolidation. Alternatively, MRD may be assessed from PB every 4-6 weeks during follow up for 24 months. | 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 end of consolidation treatment, and in PB every 4-6 weeks for 24 months after the end of consolidation. | IV | B | 94% |
| **D7** | In APL, the most important MRD endpoint is PCR negativity for *PML-RARA* at the end of consolidation. | I | A | 100% |
| **D8** | For non-high-risk APL patients, MRD monitoring is only recommended 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 months for 24 months starting at the end of treatment. Alternatively, MRD may be assessed from PB every 4-6 weeks during follow up. | -* | - | - |
| **D9** | Ongoing molecular MRD monitoring beyond 24 months of follow-up should be based on individual clinical | V | C | 95% |

| No. | Clinical MRD recommendation | LoE | GoR | LoA |
|---|---|---|---|---|
| | features. | | | |
| **D10** | Patients who are followed using 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 cycling threshold (Ct) <40 in at least 2 of 3 replicates. | III | B | 73% |
| **D13** | MRD test negativity by qPCR is defined as cycling threshold (Ct) ≥40 in at least 2 of 3 replicates, when at least 10,000 copies (but optimally ≥30,000 copies) of the housekeeping gene were measured. | II | A | 80% |
| **D14** | 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 MP-LCN) is provisionally defined as <2% but above the detection limit of the assay (ratio of the target and housekeeping genes)[80]. MRD-LL is associated with a very low relapse risk in *NPM1*-mutated patients 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. 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 detection at low level (MRD-LL). | IV | B | 93% |
| **D16** | MRD relapse is now defined as either a) conversion of MRD negativity to MRD positivity independent of the MRD technique or b) increase of MRD ≥ 1 $\log_{10}$ between | V | A | 86% |

| No. | Clinical MRD recommendation | LoE | GoR | LoA |
|---|---|---|---|---|
| | any two positive samples measured in the same tissue (PB or BM) in patients with MRD-LL. | | | |
| **D17** | 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. | IV | A | 89% |
| **D18** | Available data suggest that patients with one positive and one negative MRD result from two different techniques have a higher relapse risk than patients with two negative MRD results, but a lower relapse risk than patients with two positive MRD results. | IV | B | 95% |
| **D19** | MRD assay parameters are defined in Supplemental Table S4 and should be included in results reports. Scientific reports on MRD studies should include the parameters listed in Supplemental Table S5. | V | A | 89% |
| **D20** | Future MRD studies, including clinical trials, should report data using the thresholds and response definitions in this manuscript. | V | A | 94% |
| **D21** | Failure to achieve MRD-negative remission by MFC, molecular MRD-positivity after completion of consolidation chemotherapy, and/or MRD relapse (either molecular or MFC, as defined above) are associated with disease relapse and inferior outcomes. However, selected patients with *NPM1* mutations and CBF AML may have prolonged survival despite low-level molecular MRD (<2%, MRD-LL). | 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[84,85]; (2) MRD positive by ≥2% *NPM1* mutant copies per *ABL1* copies measured in BM or transcript levels of *NPM1* or CBF fusions failed to reach a 3-4 log reduction in the same tissue after completion of consolidation chemotherapy (ratio of target copies / *ABL1* copies between the sample at diagnosis | V | C | 100% |

| No. | Clinical MRD recommendation | LoE | GoR | LoA |
|---|---|---|---|---|
| | and the sample after completion of consolidation chemotherapy, measured in the same tissue, preferably BM);[36,71,81,86,87] and/or (3) demonstrated to have MRD relapse (either molecular or MFC), individualized treatment[84] and/or conditioning regimen strategies should be considered, preferably as part of clinical trials, in an effort to reduce disease relapse. | | | |
| **D23** | Patients with *NPM1* or CBF AML who have stable molecular MRD detection at low level (MRD-LL) do NOT necessarily require a change in treatment (at EOT 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 log10 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. | IV | B | 88% |
| **D26** | Pre-transplant 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 alloHCT myeloablative conditioning should 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% |

***No Delphi score available. Recommendation was reached after expert discussions**

**Table 2. Definitions for MRD response categories and MRD relapse.**

| Response category | Abbreviation | Defining criteria |
|---|---|---|
| **Complete remission with negative MRD** | *$CR_{MRD-}$* | 1) complete morphologic remission and<br>2) MRD negative in all MRD technologies that were used<br>a) MFC-MRD negative in BM (if MFC-MRD was used) and<br>b) qPCR-MRD negative in BM (or in PB after cycle 2 for *NPM1*- and CBF-MRD) (if qPCR-MRD was used) and<br>c) NGS-MRD negative in BM (if NGS-MRD was used) |
| **Complete remission with MRD positivity** | *$CR_{MRD+}$* | 1) complete morphologic remission and<br>2) MFC-MRD positive in PB and/or BM or<br>3) NGS-MRD positive in PB and/or BM or<br>4) qPCR-MRD positive in PB and/or BM |
| **Complete remission with molecular MRD detection at low level** | *CR-MRD-LL* | 1) complete morphologic remission and<br>2) molecular MRD detectable at low level in PB and/or BM, i.e. 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<br>2) increase of MRD copy numbers ≥ 1 $\log_{10}$ between any two positive samples in patients with CR-MRD-LL who are monitored by qPCR<br>3) The result of 1) or 2) should be rapidly confirmed in a second consecutive sample that should preferably be a BM sample. |

## Figure Legends

**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% or more blasts in 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 comparator.

Abbreviations. DfN: Different-from-Normal; LAIP: Leukemia-Associated Immunophenotype; MFC: Multiparameter Flow Cytometry; NGS: Next generation sequencing; dPCR: digital polymerase chain reaction; qPCR: quantitative polymerase chain reaction.

**Figure 2: Time points at which MRD is considered a clinically relevant biomarker.**

In figure 2 the time points and MRD cutoffs are indicated at which a MRD result may impact the therapeutic decision for a given patient. For example, in an *NPM1* mutated AML patient 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 alloHCT for this patient.

[1]After 2 cycles of chemotherapy (either 2 induction cycles or 1 induction and 1 consolidation cycle); this also includes the time point before alloHCT.

[2]Percentage *NPM1* mutant copies per *ABL1* copies measured in BM.

[3]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).

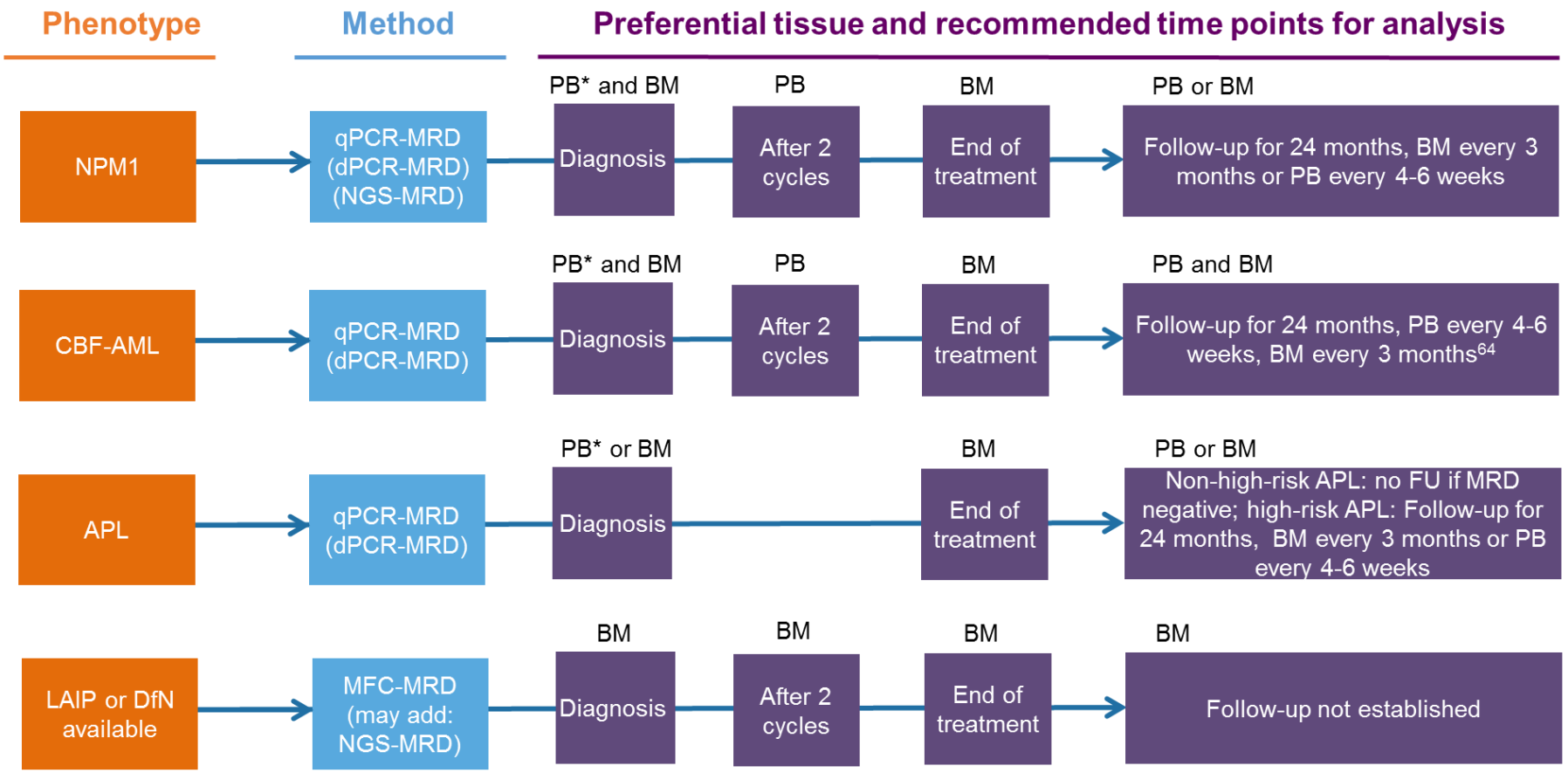


Figure 1. Heuser et al.

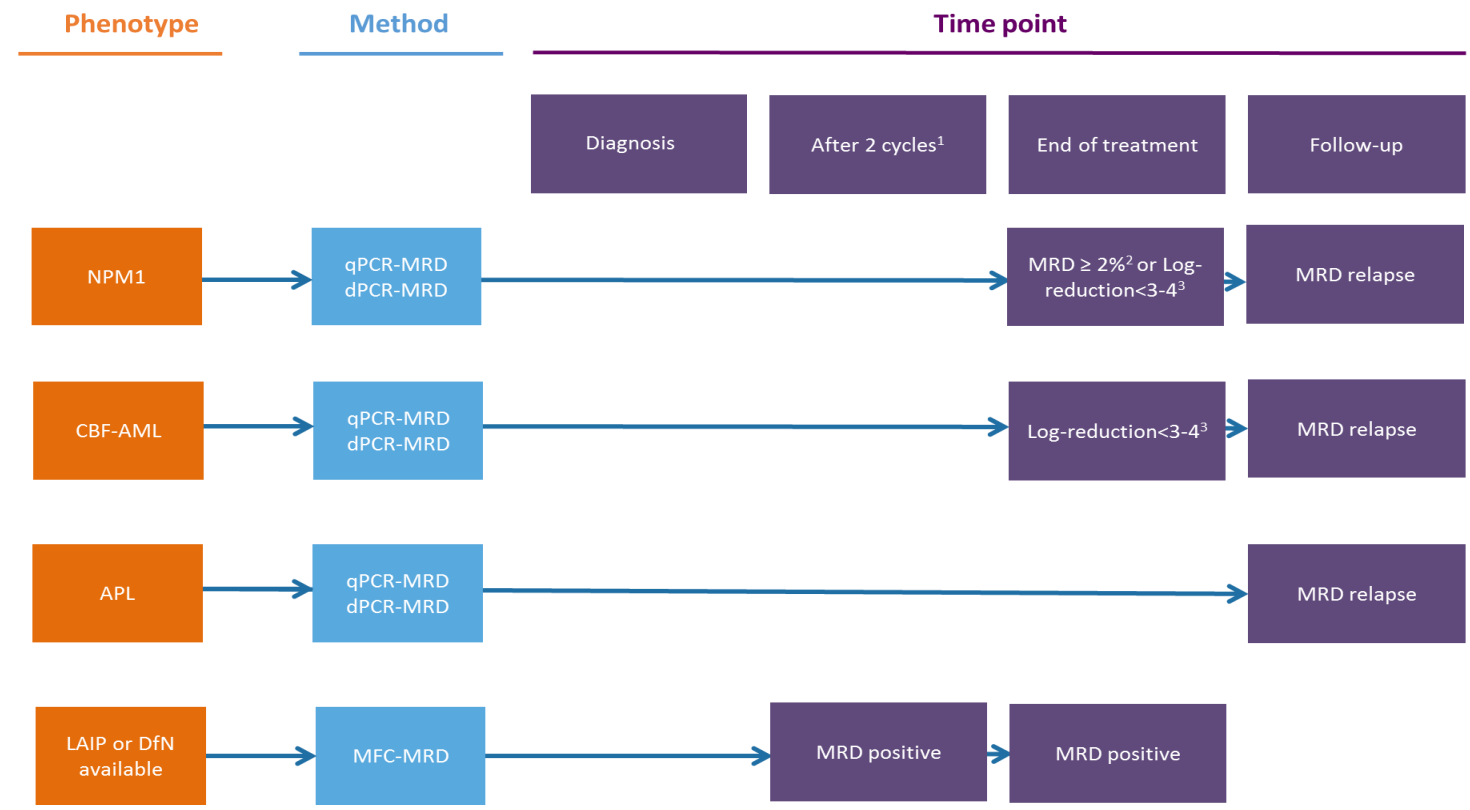


Figure 2. Heuser et al.