The present and future of measurable residual disease testing in acute myeloid leukemia

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Abstract

Considerable progress has been made in the past several years in the scientific understanding of, and available treatments for, acute myeloid leukemia (AML). Achievement of a conventional remission, evaluated cytomorphologically via small bone marrow samples, is a necessary but not sufficient step toward cure. It is increasingly appreciated that molecular or immunophenotypic methods to identify and quantify measurable residual disease (MRD) – populations of leukemia cells below the cytomorphological detection limit – provide refined information on the quality of response to treatment and prediction of the risk of AML recurrence and leukemia-related deaths. The principles and practices surrounding MRD remain incompletely determined however and the genetic and immunophenotypic heterogeneity of AML may prevent a one-size-fits-all approach. Here, we review the current approaches to MRD testing in AML, discuss strengths and limitations, highlight recent technological advances that may improve such testing, and summarize ongoing initiatives to generate the clinical evidence needed to advance the use of MRD testing in patients with AML.

Introduction

Acute myeloid leukemia (AML) encompasses a heterogeneous group of clonal neoplastic diseases of the hematopoietic system. Eradication of AML cells without intolerable harm to the production of red blood cells, white blood cells, and platelets is difficult and, combined with the often rapid proliferation kinetics, has earned this disease a reputation as one of the most challenging among all cancers to treat with a high fatality rate despite gradual improvements and expansions of therapies and supportive care measures. Intensive chemotherapy, with or without adjunctive small molecule inhibitors or immunotherapeutics, leads to a cytomorphological complete remission in a majority of AML patients. However, complete remission does not necessarily equal cure.[1] Residual AML cells may persist below the limit of cytomorphological detection and, eventually, cause overt disease recurrence. If leukemia cells capable of disease propagation are not eradicated entirely, or reduced to a level that may self-extinguish or that can be surveilled and removed by the immune system, AML will inevitably relapse.[2] Although it is widely recognized and agreed upon that residual leukemia cells, detected or not, lead to recurrent disease,[3] there is less agreement on the best ways to identify and quantify such cells, let alone how to approach them therapeutically. As a biomarker, measurable residual disease (MRD) can be both prognostic and predictive,[4] but the absolute quantifiable level of disease is not the sole determinant of patients' outcomes, as disease biology and other clinical factors modify the risk associated with MRD test results. Evidence suggests that levels of MRD compatible with long-term disease-free survival differ across molecular subtypes. Defining standards for the detection of and therapeutic approach to MRD has thus emerged as one of the major frontiers of contemporary AML management. Here, we describe residual disease in AML as a concept, the technologies currently used to detect it, limitations in this endeavor (Box 1) and provide a perspective on upcoming advancements in this area, both technological and practical.

Limits and logs

No matter whether the detection of leukemic cells is by microscopic inspection (hematoxylin & eosin staining, fluorescent *in-situ* hybridization [FISH], immunohistochem-

istry), fluorescent detection of cell surface epitope combinations (flow cytometry), or the end result of polymerase chain reaction (PCR) or high-throughput DNA sequencing, it is helpful to think of levels of residual disease as a logarithmic fraction of total normal cells measured by the same mechanism. For example, a hematopathologist may examine a bone marrow aspirate smear or biopsy core and conclude that 1%, or one in 100 cells, may be residual AML cells. Ultimately, this 10^{-2} may be the limit of detection in this context; considering a normal myeloblast cannot be distinguished reliably from abnormal via light microscopy,[5] a 5% cutoff to indicate evidence of residual disease has been established since 1956.[6] Similarly, the flow cytometrist may be able to discern one leukemic cell in 1,000 or in 10,000 cells; this limit of detection, 10^{-3} to 10^{-4}, is one to two orders of magnitude more sensitive. Nevertheless, as the total cardinality of potential comparator cells is in the billions or trillions, it follows that individually dispersed residual leukemic cells may sink below the limit of detection in the sample evaluated, but nonetheless remain a significant threat in the patient, waiting to "reemerge" to a level detectable by such testing (Figure 1). Disease "relapse" from "remission" after treatment therefore most often represents partially sensitive but refractory disease, with the rarer exceptions of cases of therapy-related myeloid neoplasm resulting from the treatment itself or true second malignancies in those with inherited or acquired predisposition to AML.

Figure 1 depicts three distinct disease and detection courses. In the first case, colored in red, disease is treated and decreases to less than 0.1% (10^{-3}), a level of sensitivity detectable with conventional flow cytometry. Until the disease relapses clinically, the patient is in an apparent flow cytometric MRD-negative remission. On the other hand, if a more sensitive flow cytometric or PCR assay with a lower limit of detection of 10^{-5} (1 cell among 100,000) were used, this patient would have been recognized as having residual disease and could have been surveilled more closely for incipient relapse or considered for MRD-directed treatments, e.g. with additional treatment such as a maintenance therapy, allogeneic hematopoietic cell transplantation, or ideally a clinical trial, acknowledging the current uncertainties about benefits and risks associated with such interventions.

Box 1. Potential reasons why current acute myeloid leukemia measurable residual disease testing is suboptimal at relapse prediction.

- ***Wrong time-point*** *– single landmark – do serial measurements help?*
- ***Wrong test*** *– MRD there but not recognized as AML?*
- ***Wrong LOD*** *– MRD there but sample incompletely characterized?*
- ***Wrong sample*** *– MRD in patient but not in the tube? (See #1 above)*
- ***Wrong premise*** *– Who says MRD testing will detect 100% of relapses?*

MRD: measurable residual disease; AML: acute myeloid leukemia; LOD: limit of detection.

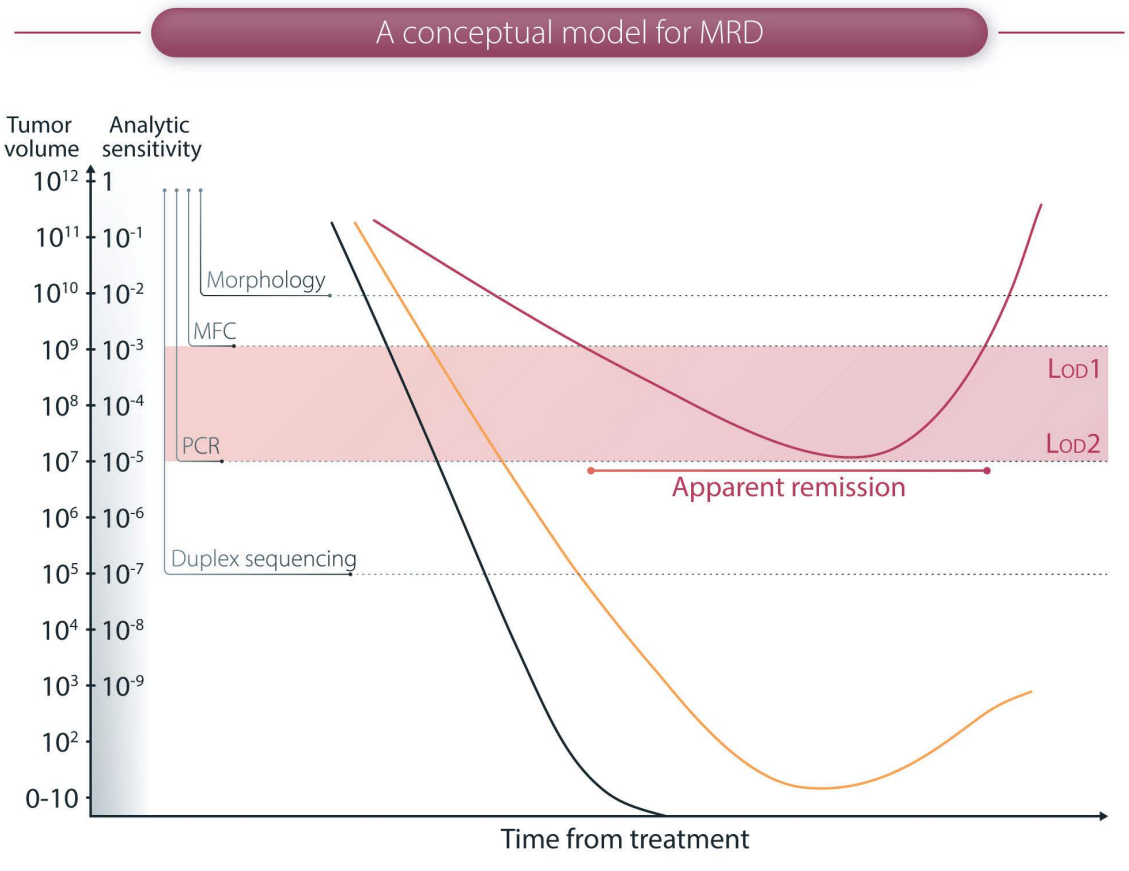

Figure 1. A conceptual model for measurable residual disease. Quantifiable disease, shown as three colored lines, red, orange, and black, may take very different trajectories. In red, an apparent remission (see text) is followed by a rapid relapse. In orange, undetectable disease might relapse later, or be extinguished by host immunity. In black, a cure. MRD: measurable residual disease; MFC: multiparameter flow cytometry; PCR: polymerase chain reaction; LOD1: limit of detection for technology 1. LOD2: limit of detection for technology 2.

The patient whose disease burden is drawn in orange, on the other hand, had levels of residual disease largely unmeasurable by currently available MRD assays, in which a sensitivity of one in 10^5 or less is most typical. With such assays, this patient then had no *measurable* residual disease. From this low level of disease, relapse may occur at any later point in time, or cure may result, e.g., through additional chemotherapy and/or an immune effector cell. Finally, we could imagine a patient (exemplified in black) who received treatment effective enough to eradicate 100% of existing tumor cells. However, it is essential to recognize that we cannot draw a distinction between patients orange and black without the availability of more sensitive MRD assays. In the next section, we describe various techniques for the detection of MRD, and their analytic and practical limits (Figure 2).

Current state of measureable residual disease testing in acute myeloid leukemia

Detection, or *measurement*, of MRD is a sine *qua non*, but the specific measurement mechanism profoundly affects analytical sensitivity, with different methods having limits of detection ranging over several logs. This assay-sensitivity dependence, and the recognition that a negative test does not imply the absence of disease, is a primary reason why the field has shifted from the term "minimal residual disease" to "measurable residual disease" in the last few years.[7]

After 60 years of relative stasis in the response criteria used for AML,[8] in 2017 the European LeukemiaNet (ELN) introduced a new category of MRD-negative complete re-

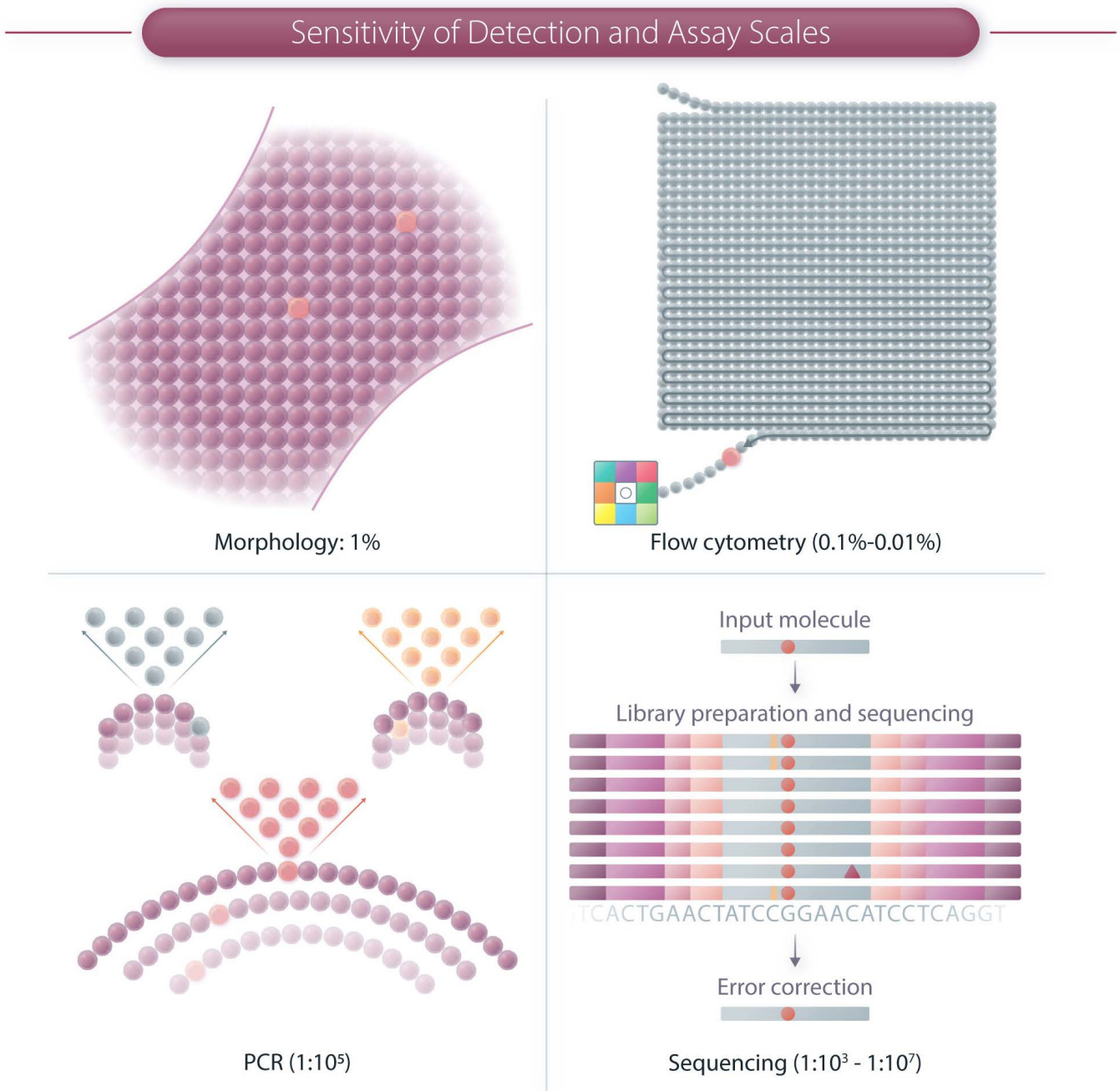

Figure 2. Sensitivity of detection depends on technology and technique. Upper left. Morphological examination of the marrow, metaphase karyotypes, or fluorescent *in-situ* hybridization is limited to a sensitivity of about 1%. Upper right. Multiparameter flow cytometry with carefully chosen markers can achieve a sensitivity of 10^{-3}, or acute myeloid leukemia constituting 0.1% of events. Experts using extensive panels can go lower. Lower left. Polymerase chain reaction (PCR), whether normalized to a reference transcript such as *ABL1* or quantified absolutely with digital PCR can look deeper, between 10^{-4} and 10^{-5}. Lower right. While conventional next-generation sequencing has an error rate of around 0.1%, error correction technologies can markedly improve the limit of detection to better than 10^{-7}.

mission in their 2017 guidelines update in acknowledgment that patients who test MRD negative have better outcomes than those who test positive after receipt of the same therapy.[9] This was followed in 2018 by the release of the first ELN consensus standard-of-care guidelines containing detailed specifics on flow cytometry, molecular testing, and clinical aspects of AML MRD.[10] Molecular testing by a validated quantitative PCR test was recommended for those patients with an AML containing a stable and characteristic genomic aberration, namely acute promyelocytic leukemia, core binding factor leukemias, rare *BCR-ABL* translocated AML, or those with the canonical *NPM1* insertion. For the approximately 60% of patients without such a mutation to track, the recommendation was - and remains - multiparameter flow cytometry (MFC). The NCRI AML17 study group demonstrated in a large study of 2,450 patients that MFC can be an effective discriminator in select patients,[11] and meta-analyses suggest that it is broadly applicable.[12] In 2021 the ELN AML MRD guidelines were updated (with the expectation that these evidence-based guidelines will be iteratively updated every 2-4 years as new data become available) with nearly 60 refined recommendations spanning across MFC MRD, molecular MRD, clinical use of MRD, and directions for possible future improvements.[13] New recommendations were also included for the use of next-generation sequencing in AML MRD detection (NGS-MRD).

In contrast to its sibling leukemias, AML is descended from a transformed cell that bears no universally unique molecular signature. Contrasting with *IGHV* or *TCR* gene rearrangements that reliably mark a clonal lineage of acute lymphoblastic leukemia cells, the AML genome is more heterogeneous and does not afford the opportunity for a single all-encompassing assay (viz., *IGHV*-directed PCR) as in acute lymphoblastic leukemia.[14] While it is currently unproven for these most sensitive of AML MRD assays what is the optimal combination of targets for monitoring, it is clear that not all mutations found at initial AML diagnosis will have equal clinical utility for MRD monitoring.[15,16] The optimal targets for molecular MRD measurement have also not been defined beyond the recognition that the isolated detection of a mutation found commonly in age-related clonal hematopoiesis (e.g., *DNMT3A*, *TET2*, or *ASXL1)*[17–20] or in germline predisposition syndromes (e.g., *DDX41*, *RUNX1*, or *GATA2*) does not necessarily represent residual AML (Figure 3). Moreover, existing data indicate that mutations in signaling pathway genes (*FLT3*, *KIT*, *RAS,* etc.) are useful when positive, but as later clonal acquisitions, should not lead to false reassurance if negative, perhaps particularly when the mutation is therapeutically targeted (Table 1). Ultimately, accumulated knowledge about each molecular marker's utility and tradeoffs could inform a patient-directed, multi-mutation testing strategy.[21]

Acknowledging that the result of any given MRD test may have a different significance (including no negative impact) in different clinical situations, an important area of current thought is the definition of optimal monitoring strategies

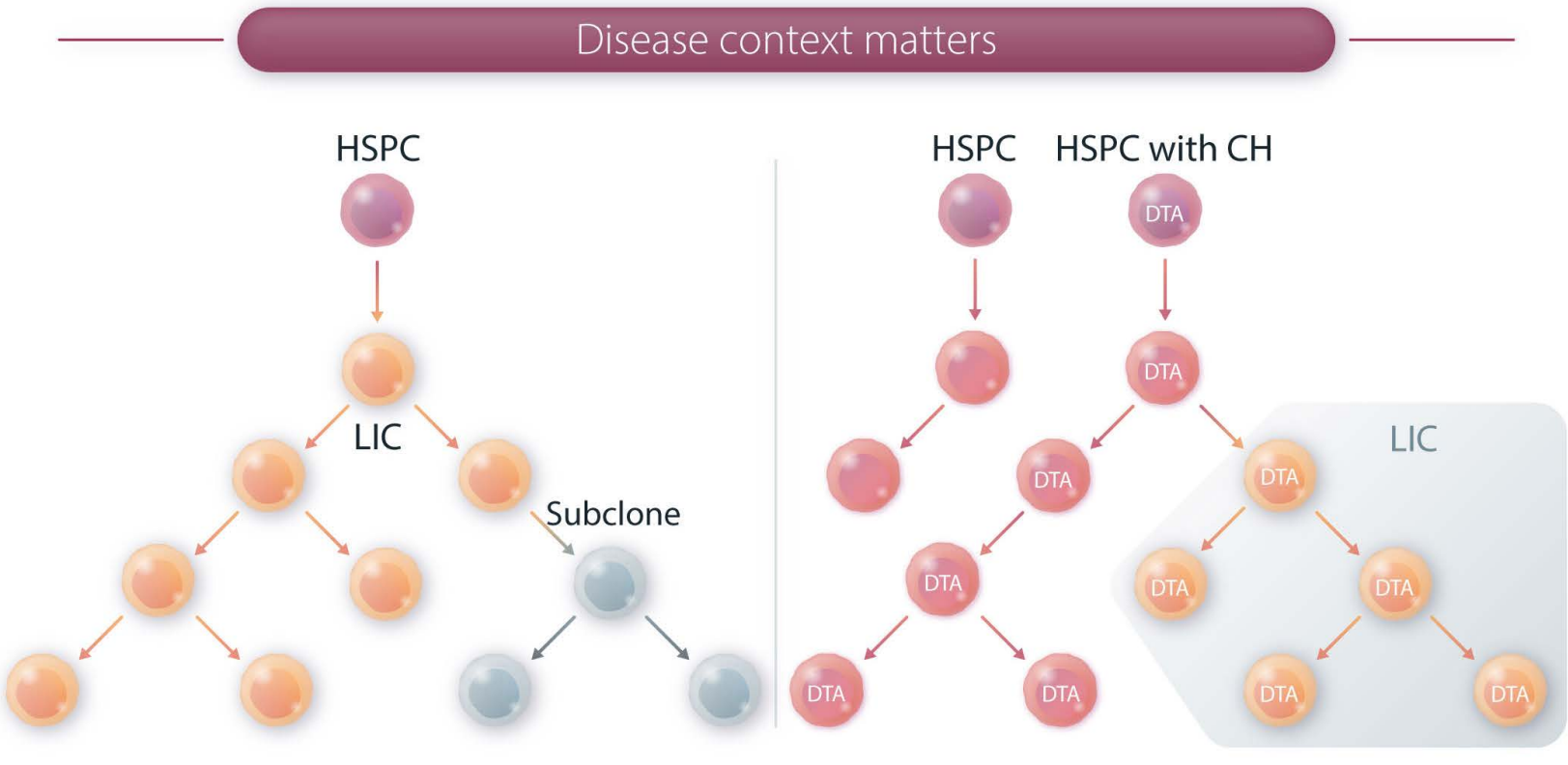

Figure 3. Disease context matters. Left. Tracking the subclone in blue may not represent the actual leukemia (orange). Disappearance of the subclone could lead to wrong conclusions about remission status. The best measurable residual disease surrogate is often, but not always, the most primordially acquired mutation. Right. Clonal hematopoiesis complicates this picture. Mutations in *DNMT3A*, *TET2*, and *ASXL1*, as well as other genes, may exist above the level of leukemic transformation within the hematopoietic hierarchy and complicate tracking of residual disease. HSPC: hematopoietic stem and progenitor cell; LIC: leukemia-initiating cell; CH: clonal hematopoiesis.

Table 1. European LeukemiaNet-approved molecular acute myeloid leukemia measurable residual disease targets.

Genetic change	Recommended in ELN AML MRD 2021 or ELN 2022	Comments
NPM1	Yes	Essential to inform postremission therapy.[65-67]
RUNX1-RUNX1T1 or *CBFB-MYH11*	Yes	Measurement of fusion transcripts by qRT-PCR highly informative, but there is evidence that ultra-low levels of disease can be serially monitored.[34,68–72]
Other fusions such as KMT2A, DEK-NUP214,[73] MECOM	Not specifically	Smaller bodies of evidence but concept is sound. *KMT2A* has over 40 partners.[74]
Signaling pathway genes: *FLT3, KIT, RAS*, others	Possibly	Useful if positive but relapse possible in test-negative subjects.
"DTA" genes: *DNMT3A, TET2, ASXL1*	Specifically recommended against	These may be found in age-related clonal hematopoiesis and should be excluded from consideration.[17-20]
Hereditary predisposition genes: *ANKRD26, CEBPA, DDX41, ETV6, GATA2, RUNX1,[75] TP53*	See comment	When present at a variant allele frequency of ~50%, specifically exclude from consideration.
WT1, *EVI1*	Disfavored	Expression-based assays may be highly variable.[76-89]

ELN: European LeukemiaNet; AML: acute myeloid leukemia; MRD: measurable residual disease; qRT-PCR: quantitative reverse transcriptase polymerase chain reaction.

for individual patients, taking into account their unique disease and treatment contexts. The impacts of MRD testing (and possible subsequent action) are situationally dependent; thus, data collection and recommendations may need to be individualized. This may include genetic context (Table 1) but also risk group[11] and allogeneic transplantation status.[12,22,23]

Finally, in addition to the assay itself, a key ingredient in sensitivity is supplying the assay with an appropriate input. Typically, cells are obtained from bone marrow or peripheral blood samples, or fluids/tissues of other organs. A hemodilute, hypocellular specimen may defeat the purpose of cell-based analytical techniques and cannot be considered sensitive. However, emerging data suggest that molecular analyses of liquid biopsies may have value for MRD testing in AML. Nakamura and colleagues found that cell-free, circulating tumor DNA may even have higher prognostic value than bulk peripheral blood in some contexts.[24] Further studies will be needed to determine what role cell-free DNA testing will play in a hematologic malignancy for which tumor-containing compartments can be accessed relatively easily.

Morphology

⟹ *Key point. Cytomorphology, sensitive only to about 1-5%, remains a standard part of complete remission criteria and is widely available.*

Morphological examination of the bone marrow is the oldest and best-established technique for the quantification of myeloid leukemia. Typically, cross-sections of bone marrow core biopsies stained with hematoxylin-eosin are visually inspected, and, if needed, stained with antibodies and immunohistochemistry reagents for more precise cell identification. Bone marrow aspirates are smeared, affording more space between distinct cells, and subjected to Wright stain. Finally, some portion of the aspirate is often cultured for visualization of G-banded metaphase karyotypes and FISH studies. In direct examination of cells and in FISH analysis, about 200-500 total cells will be examined and tabulated with their identity. Clearly, the sensitivity on average could be no greater than 1:200-1:500, or 0.2-0.5%, but given statistical constraints and normal variation, it is reasonable to conclude that the limit of detection is in the order of 1%.

Because of the ubiquity, general sensitivity, and inexact art of morphological examination,[5] a threshold of 5% myeloblasts was established more than 60 years ago[6] as a cutoff for complete remission in conjunction with blood count recovery. This legacy persists today, with national and international expert consensus groups continuing to include <5% bone marrow blasts as a central complete remission criterion.[25,26] In FISH studies, the definition of abnormal varies on a per-probe basis, but typically lies between 1-5%. Although FISH improves upon microscopy by offering evidence of specific leukemia-associated changes that are definitively not present in normal myeloblasts, many AML cases have a normal karyotype[27] (i.e., there is nothing to detect by FISH), and the technique is further limited by cost and throughput. Overall, advancement beyond this will require similarly ubiquitous assay(s) with broad applicability to many (ideally, all) AML patients and read-outs that are non-ambiguous to interpret quantitatively. As we will see, each of the more modern techniques remain wanting in some way.

Immunophenotyping by multiparameter flow cytometry

⇒ *Key point. Considerable expertise is required for correct interpretation of acute myeloid leukemia measureable residual disease flow cytometry using a leukemia-associated immunophenotype-based difference-from-normal approach.*

⇒ *Key point. While recommendations for monoclonal antibody panel compositions have been provided, "off-the-shelf" standardized pre-mixes are not yet widely available.*

Immunophenotyping by MFC is a key tool to establish a diagnosis of AML and has proven useful for the detection of MRD. Historically, two separate approaches have been developed as bases for MRD assays, one focusing on the identification of one or more leukemia-associated immunophenotypes (LAIP) at diagnosis that are then tracked throughout the treatment course, and the other focusing on the identification of cell population(s) showing deviation(s) from antigen-expression patterns typical of normal or regenerating cells of similar lineage and maturation stage ("difference from normal [DfN]").[10,13,28,29] Used in isolation, the LAIP approach may lead to false negative testing because of immunophenotypic shifts throughout the course of AML, whereas the DfN approach may be particularly susceptible to false positive interpretation. Acknowledging such limitations, it is currently recommended to combine these two approaches ("LAIP-based DfN approach") for the monitoring of diagnostic and emergent leukemic clones.[13]

Advantages of MFC-based MRD assays are their wide applicability with suitability for >90% of all patients with AML if a comprehensive panel of monoclonal antibodies is used, relative ease of quantifying abnormal cell populations, rapid turn-around times, assessment of hemodilution, ability to distinguish live from dead cells, and the possibility to identify immunotherapy targets. As significant limitations, not all cases of AML have an abnormal immune phenotype and/or phenotype(s) may change over time, the sensitivity of the assay is not uniform between patients, fresh material is required for best results, and analysis/data interpretation typically includes subjective elements. Perhaps most importantly, MFC-based MRD assays require experience and expertise, and assay standardization or even harmonization has proven challenging. Development and validation of (somewhat simplified) antibody panels that enable a harmonized MFC-based MRD assessment is an important focus of ongoing work to advance this methodology.[29] We and others have evaluated compartmental differences and found that MFC-based MRD testing of peripheral blood samples may yield similar findings to those obtained with testing of bone marrow specimens, with sensitivity and specificity ≥90% in most cases.[30] Additional, ideally prospective, comparative evaluations to expand on this work will be helpful to further refine the relative values of peripheral blood *versus* bone marrow MFC MRD testing. Other areas for potential advances involve automated approaches to the analysis and interpretation of MFC MRD data,[13] and determination of the potential value of including evaluations of less mature ("leukemia stem cell") populations in MRD assays. While such approaches entail intrinsic challenges (e.g., with regard to the exact definition, or sorting strategy) of "leukemia stem cell" populations, emerging data indicate that considering such cell populations might refine risk assessment relative to conventional MFC MRD testing.[31,32]

Quantitative polymerase chain reaction

⇒ *Key point. Polymerase chain reaction requires a specific target mutation or fusion for which to search.*

⇒ *Key point. Polymerase chain reaction can detect DNA mutations at very sensitive levels; its use for RNA gene expression quantification in measurable residual disease is disfavored. Detection of fusion genes from RNA input remains very valuable.*

⇒ *Key point. Digital polymerase chain reaction is emerging as a potentially more sensitive and standardizable way to quantify specific mutations absolutely.*

The PCR recursively amplifies billions of copies of an original starting template molecule. This sounds like an ideal molecular technology for the detection of low levels of residual AML, but it is complicated by a number of factors. The first is the heterogeneity of AML: while there are scores of different mutations and cytogenetic abnormalities, the genome itself is relatively stable (except in late stages and in some subtypes such as *TP53*-mutated or complex karyotype AML), with among the lowest tumor mutational burden of all cancers studied in a landmark pan-cancer analysis.[33] Functionally and practically, AML is dozens of different diseases, and a specific PCR test or panel must be selected and individualized to each patient. On the other hand, PCR is highly specific and widely available, both important characteristics for a test performed in the remission setting.

A second potential difficulty with PCR as an aberration-specific assay is that it provides only a relative quantification of targets in the analyte. A PCR test is determined to be positive when a fluorescent signal is detected above some pre-specified threshold after a certain number of cyclic amplifications. This cycle number as a power of two (i.e., 2^{Ct}) provides information about the original amount of detected mutation (or fusion transcript, etc.), but only in terms of a relative copy number. Typically, this limitation is overcome by normalizing against a standardized comparator such as *ABL1* transcripts. Overall, the lack of absolute quantification in quantitative reverse transcription PCR (qRT-PCR) complicates standardization and studies of distinct levels of MRD.

Digital PCR (dPCR) is a relatively newer technique that is

based on the fundamental principles of PCR but differs from qRT-PCR in that it provides an absolute quantification of molecules, which can then be normalized to total volume of source analyte (microliter of blood, etc.). dPCR is sometimes referred to as "droplet digital PCR," as the first commercially successful dPCR assays relied on partitioning an input sample, and its constituent DNA, into thousands of microscopic oil-water emulsion droplets. These droplets then each host individual PCR reactions, and the absolute proportion of detected target molecule could be calculated discretely ("digitally") by dividing the number of positive droplets by negative droplets. This absolute quantification has immense advantages: assays are easier to standardize across laboratories, are not dependent on fluctuations in the normalization target, and the results are more directly interpretable by clinicians and researchers. On the other hand, even with such "standardization," the cutoff between positive and negative tests relative to a noise floor, as well as a quantitative level that is actionable remains to be established and must be validated in clinical settings. What is more, the relevant standards differ for different molecular targets (Table 1), and compartments (marrow *vs*. blood) can have a 100-fold impact on detectability of some targets.[34] Finally, clinicians must be mindful that even when dPCR is an excellent fit for their patient and disease context, there are important limitations. For example, dozens of distinct *NPM1* mutations exist, but three (types A, B, and D) account for about 90% of cases.[35–37] dPCR assays that cover only the most common three could lead to a wrong conclusion of MRD negativity for a patient with a type C mutation that was discovered via NGS, for instance. Overall, the sensitivity of dPCR is similar to that of traditional qRT-PCR, between one in 10^5 and 10^6, but because of its advantages, dPCR will likely supplant the other assay over time.

Sequencing

⟹ *Key point. DNA sequencing is both the most versatile and sensitive technique, but standards and advancements are still being established.*

⟹ *Key point. The error rate intrinsic to conventional next-generation sequencing makes false positive measurable residual disease calls likely, but laboratory and bioinformatic error-correction techniques can reduce this risk and are strongly recommended.*

NGS, also called high-throughput sequencing, is one of the most transformational technologies in biology and medicine in the last 20 years.[38] The ability to assay a large number of genes, a small number of genes extremely deeply, or – limited only by resources – a large number of genes deeply, as well as quantifying either DNA or RNA (including gene expression and fusion detection) has generated considerable enthusiasm for NGS-based technology for MRD testing in AML.[39]

High-throughput sequencing leverages PCR, plus advances in microfluidics and photonics/optics to effectively miniaturize and repeat on the scale of billions of times a conventional Sanger reaction. The individually sequenced fragments can be computationally reassembled into stacks of reads representing a digitally quantized representation of the assayed allele pool. Researchers or clinicians can then examine genomic positions of interest to estimate the fraction of mutant DNA in the sample.

This is an exciting and powerful technique for the assessment of residual AML for several reasons. First, NGS has become a commodity oncology product, widely available at individual hospitals and with numerous send-out options – although turnaround time remains a concern in the acute context of AML. Secondly, it greatly simplifies the burden on the clinician who can order a single panel covering multiple pathogenic mutations, albeit at higher cost. The biological limitation in this is that AML is the perfect storm of low mutational burden,[33] lacking a unifying or pluralistic driver, and frequently possessing genomic aberrations that are not single point mutations (i.e., fusions and other structural variations) which are more difficult to detect with current NGS approaches. Consequently, even a broad NGS panel may not effectively query many types of AML genomes.

There is also a technical limitation as we seek to look deeper: clinical assays often do not report variants detected with a frequency lower than 1% due to the error rate of conventional NGS resulting in the risk of false positive results. This means that while we might be able to make an estimate of mutant allele fraction ~0.5% when we observe 50 variant reads in 10,000, we cannot simply 10X the coverage of a particular hotspot each time we want another log of sensitivity beyond this. To address these limitations, which primarily stem from propagated PCR errors (Figure 2, lower right), a variety of error suppression approaches have been introduced. Duplex sequencing[40,41] is arguably the most specific of these methods, capable of reducing error rates to a point at which one mutant allele among 10^8 wild-type alleles can be correctly detected. Unfortunately, these techniques require sophisticated laboratory and bioinformatics personnel and are not yet widely available in 2022, although a number of companies have introduced a variety of error-correction kits and we expect the number of academic and commercial offerings to grow quickly. The necessity of reducing error rates compared to NGS assays used at diagnosis, particularly for the detection at MRD level of single nucleotide variants, has resulted in the 2022 ELN guidelines now making a specific recommendation for error-corrected sequencing,[26] and major studies in AML have now incorporated it.[15,16,19,42–44]

NGS-based methods may represent the future for AML MRD testing with promises of assay reproducibility, highly

quantitative nature, and the potential to harmonize testing and interpretation across sites. Despite this, there is currently insufficient evidence to recommend NGS-MRD as a standalone AML MRD technique.[26] The recommended limit of detection for NGS-MRD testing in AML, as for multiparameter flow cytometry and other molecular testing, is *at least* one in 1,000, although the optimal threshold level that best discriminates subsequent relapse risk has not yet been defined for individual mutations, combinations of mutations, or treatment time-points. There is also evidence that thresholds lower than 0.1% can significantly increase sensitivity with only a small loss in specificity.[30]

Novel approaches

There are a variety of potential uses for results from AML MRD assays, including monitoring for relapse detection after treatment, quantification of treatment response, identification of patients in remission but at high risk of relapse at key clinical landmark time-points (i.e., for clinical trial enrollment), and potentially as a surrogate endpoint for overall survival.[45] MRD assays may be most immediately useful in informing the risk of relapse, a notion supported by findings from large meta-analyses.[3,12] However, at the level of individual patients, our ability to predict relapse is suboptimal,[46,47] cautioning against overreliance on a negative MRD test result to guide decision-making and prognostication. Current MRD testing is limited for many potential reasons (Box 1). Numerous new technologies and approaches are, however, under development to further improve MRD detection and thus early warning of relapse. To overcome technological and physical limitations previously mentioned (Box 2), many of these focus on pre-enrichment of target cells. In the RELAZA study, Platzbecker and colleagues sorted $CD34^+$ cells for donor chimerism analysis to select patients for maintenance intervention in anticipation of relapse.[48] The same investigators also used $CD34^+$ enrichment followed by sequencing to effectively detect MRD in non-transplant AML patients with high sensitivity and specificity, and significantly earlier than un-enriched NGS.[49] We have also shown that enhancing the sorting process for a leukemia-initiating cell phenotype provides the ability to interrogate the earliest and smallest compartments responsible for relapse.[50] Clearly, dedicated affordable instrumentation to assist in these techniques would need to be developed before pre-enrichment could find more widespread use.

Sequencing-based single-cell technologies afford the opportunity to combine informative elements of surface immunophenotype with genetic mutations. Current investigations using joint profiling have been focused on enhancing the understanding of biology from multimodal data,[51,52] but future work could leverage these learnings to dissect patients with lower *versus* higher risk of relapse in the setting of *DNMT3A*, *TET2*, and *ASXL1* (DTA)-type or IDH mutations according to the expression of higher risk markers on subpopulations.

Other technological advancements that will impact practical aspects of MRD testing concern not necessarily the depth of interrogation, but the time and place. Different AML subtypes have different relapse kinetics.[53] As schematically depicted in Figure 1, residual leukemic cells below the limit of detection may eventually gain replicative momentum and return, which may occur between visits.[53] Longitudinal testing[4,54] in settings out of major leukemia centers, including the home, could facilitate earlier detection and intervention. Small, portable sequencing instruments that can be deployed in remote settings[55] and have already been demonstrated to measure mutations in

Box 2. Can we go lower?

Besides the biological and technical limitations described in the text, there is a physical limitation. The denominator in mutant allele detection is normal genomes, or cells. To achieve 1:10^9 sensitivity practically requires 3x10^9 inputs. At a cellular concentration of 10,000/μL, this requires a sample of 100-300 mL, clearly an impractical enterprise. At 1,000/μL, it is absurd to consider. The figure depicts the theoretical approximate sampling volume (bottom row) required for a given limit of detection (top row) assuming 1,000 nucleated cells per microliter. Incorporation of cell-free DNA is one way to overcome this; continuous surveillance[54] at a lower level is another possibility.

Box 3. Basic and answerable questions regarding acute myeloid leukemia measurable residual disease testing.

- *What is the degree of discordance between AML MRD measured by FC and NGS?*
- *Can blood substitute for marrow in flow/NGS AML MRD assessments?*
- *Are all/any detected non-DTA mutations appropriate for NGS tracking in remission? – Or are some more pathognomonic than others?*
- *What about residual genetic constituents of an antecedent MDS?*
- *How often are subclones responsible for relapse found in remission and/or in the original diagnostic sample when using highly sensitive MRD-depth NGS measurements? i.e.: can we predict potential escape clones?*
- *Does AML MRD negativity have the same prognostic significance if achieved after intensive vs. non-intensive therapy?*

AML: acute myeloid leukemia; MRD: measurable residual disease; FC: flow cytometry; NGS: next-generation sequencing. DTA: DNMT3A, TET2, ASXL1; MDS: myelodysplastic syndrome.

AML[56] are an exciting development. At-home blood collection is another area that has seen recent increasing development.[57] Widespread availability of at-home blood collection and/or miniaturized, economical sequencers might enable more frequent monitoring with a risk-adapted schedule when the point-of-care is not a major leukemia center or from the patient's own home.

Ongoing initiatives to generate evidence for measurable residual disease testing in acute myeloid leukemia

As previously discussed, the ELN produces evidence-based, international consensus clinical standard-of-care guidelines for the use of MRD testing in AML.[10,13] In addition, guidance for industry is available from the US Food and Drug Administration.[58] Both these documents are limited however due to the lack of available high-quality evidence regarding specific tests, contexts of use, and clinical utility. Many important fundamental questions remain (Box 3). In the context of this deficiency several new national or international efforts have been initiated to generate evidence supporting the use of MRD testing in AML.

While AML MRD testing for response assessment now represents the standard of care and is therefore integrated into most high-quality clinical trials, the clinical evidence basis for the next generation of AML MRD tests, based on genomic assays as described above, is still being developed. Planned for launch in late 2022 or early 2023, myeloMATCH is the North American precision medicine master protocol initiative for patients with myeloid malignancies. This program, a collaboration between the National Cancer Institute and the Cancer Cooperative Groups (ECOG-ACRIN, SWOG, The Alliance, Canadian Cancer Trials Group, Children's Oncology Group), aims to assign patients to randomized controlled trials at each stage of therapy. While initial therapy allocations will be made using baseline genomic and clinical characteristics, the intent is to use AML MRD results as both an endpoint of trials, but also the inclusion criterion in randomized controlled trials for subsequent lines of therapy (i.e., consolidation, transplant, maintenance). MRD will be tested by flow cytometry, but also by novel molecular methodologies such as ultra-deep DNA-sequencing on both blood and bone marrow at key clinical landmarks.

In addition to myeloMATCH, there are also several other ongoing or planned programs of importance to the clinical development of AML MRD testing. The Foundation for the National Institutes of Health (FNIH), established by Congress in 1990 is a not-for-profit 501(c)(3) charitable organization that works with partners to accelerate biomedical research. In early 2022 the FNIH announced the launch of the public-private-academic AML MRD Biomarkers Consortium, including the National Institutes of Health, Food and Drug Administration, and over 20 private sector research, diagnostic, or pharmaceutical industry partners. The stated goals of this consortium are to generate reference standards for AML MRD, compare molecular methods for AML MRD detection, generate evidence of clinical utility using retrospective bio-banked material, and facilitate generation of prospective evidence in ongoing or upcoming clinical trials. An earlier industry-led group, MPAACT (*Measurable residual disease Partnership and Alliance in Acute myeloid leukemia Clinical Treatment*), with origins dating back to at least 2018, reports a focus on establishing MRD as a surrogate endpoint for overall survival in the treatment of AML. This work, to be performed by the Mayo Clinic Statistics and Data Management Center, would involve a meta-analysis to assess the association of MRD with overall survival based on data from across multiple clinical trials.[59] A large European initiative named HARMONY (*Healthcare Alliance for Resourceful Medicine Offensive against Neoplasms in Hematology*) is a big data program of 100 organizations from 18 European countries with data from over 100,000 patients with hematologic malignancies. HARMONY proposes, in an analysis called "AML-4" of over 7,500 AML patients, to evaluate MRD status after two cycles of chemotherapy as a potential surrogate endpoint for overall survival in AML. This will include analysis of flow

cytometry and molecular (*NPM1* quantitative PCR) test results, measured in blood and marrow, of patients treated in randomized controlled trials of initial AML treatment.[60] Two related, large US-based efforts, each reporting on approximately 1,000 patients, will focus on the association between overall survival in patients with AML in remission undergoing allogeneic hematopoietic cell transplantation and the results of genomic MRD testing before (Pre-MEASURE)[61] and after (MEASURE, NCT05224661) transplantation.

Discussion

AML has consistently been at the forefront of cancer genetics and genomics.[62,63] In contrast, it has lagged behind the three other major types of leukemia in sensitive and standardized tests for low levels of disease, at least in part because of its molecular and immunophenotypic heterogeneity (i.e., lacking an analogue to *IGH*, *TCR*, or *BCR-ABL*). In some ways, our standards and expectations of MRD detection remain stuck in a decades-old rut, just as our treatment paradigms were until the targeted therapy revolution began in 2017.[64] Newer developments, including sophisticated multicolor flow cytometry assays, a proliferation of target-specific PCR assays, error-corrected DNA sequencing, and the incorporation of these advanced methods in large studies have led to a new era for the management of AML, but many opportunities and questions remain (Box 3).

What further developments in technology and understanding of disease biology will have the highest yield in terms of improved patient outcomes? We are particularly excited about the potential for sequencing-based techniques to interrogate both deeply and broadly across a variety of lesions for a continually decreasing cost. The prospect of longitudinal home-based testing could overcome the problem of single point-in-time snapshots. Large patient cohort studies, such as HARMONY and MEASURE, are crucial to provide the basis for new knowledge and insights. Focused partnerships between experts in disease assessment and therapeutic intervention, as in the MRD-focused arms of myeloMATCH, will be essential to translate learnings.

Finally, and most pressingly, how can we make present advancements more standard and widely accessible? The convening of groups such as the ELN MRD Working Party and the promulgation of expert consensus recommendations is a critical first step, but the molecular heterogeneity of AML makes the practical implementation of these recommendations difficult. Commoditization of a complex decision tree, whether ultimately through centralization or simplification and dissemination, will be required for all AML patients to benefit from these advances.

Disclosures

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