REVIEW

Criteria for Diagnosis and Molecular Monitoring of *NPM1*-Mutated AML

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ABSTRACT *NPM1*-mutated acute myeloid leukemia (AML) represents the largest molecular subgroup of adult AML. *NPM1*-mutated AML is recognizable by molecular techniques and immunohistochemistry, which, when combined, can solve difficult diagnostic problems (including identification of myeloid sarcoma and *NPM1* mutations outside exon 12). According to updated 2022 European LeukemiaNet (ELN) guidelines, determining the mutational status of *NPM1* (and *FLT3*) is a mandatory step for the genetic-based risk stratification of AML. Monitoring of measurable residual disease (MRD) by qRT-PCR, combined with ELN risk stratification, can guide therapeutic decisions at the post-remission stage. Here, we review the criteria for appropriate diagnosis and molecular monitoring of *NPM1*-mutated AML.

Significance: *NPM1*-mutated AML represents a distinct entity in the 2022 International Consensus Classification and 5th edition of World Health Organization classifications of myeloid neoplasms. The correct diagnosis of *NPM1*-mutated AML and its distinction from other AML entities is extremely important because it has clinical implications for the management of AML patients, such as genetic-based risk stratification according to 2022 ELN. Monitoring of MRD by qRT-PCR, combined with ELN risk stratification, can guide therapeutic decisions at the post-remission stage, e.g., whether or not to perform allogeneic hematopoietic stem cell transplantation.

INTRODUCTION

NPM1-mutated acute myeloid leukemia (AML) represents the largest molecular subgroup of AML in adults, accounting for 30% to 35% of cases (1). *NPM1* mutations are driver genetic events that are AML-specific (2) and promote leukemia acting in concert with mutations of other genes, usually associated with clonal hematopoiesis, such as *DNMT3A*, *IDH1/2*, and *TET2* (2). *NPM1* mutations are characterized by the aberrant cytoplasmic localization of the NPM1 mutant (NPM1c^{+}; refs. 1, 3) and a unique gene-expression profile (2). Because of its unique features (Table 1), *NPM1*-mutated AML is recognized as a distinct leukemia entity in both the 2022 International Consensus Classification (ICC; ref. 4) and World Health Organization (WHO) 5th edition (5) classifications of myeloid neoplasms.

The aberrant accumulation of NPM1c in the cytoplasm of AML cells (1, 3) plays a key role in leukemogenesis (2). This is supported by the observation that all NPM1 mutants, regardless of the affected exons (3, 6), lead to the cytoplasmic localization of nucleophosmin. In addition, all *NPM1* mutations are "born to be exported" since the nuclear export activity of NPM1c is strictly regulated by the strength of the C-terminal nuclear export signal (NES; ref. 3). Moreover, NPM1c is critical for HOX gene expression and leukemic state maintenance (7). Additionally, a gain-of-function in the cytoplasm, leading to the inhibition of caspase-6 and -8 with deregulation of cell death and myeloid differentiation, has been reported (8), and NPM1c was found to hamper the formation of promyelocytic leukemia nuclear bodies, which are regulators of mitochondrial fitness (9). However, the function of NPM1c in the cytoplasm still remains elusive.

NPM1c is recruited to chromatin through Exportin-1 (XPO1) (10) and controls HOX/MEIS expression (11, 12). HOX/MEIS overexpression can be blocked using either menin or XPO1 inhibitors. Menin inhibitors disrupt the menin–MLL1 network (11) and show strong antileukemic activity in *KMT2A*-rearranged and *NPM1*-mutated AML, both *in vitro* and *in vivo* (13). The menin inhibitor revumenib led to 21% complete remissions in *NPM1*-mutated AML patients, often with measurable residual disease (MRD) negativity, downregulation of MEIS and HOXA9 genes and increased expression of CD14 (14). XPO1 inhibitors cause the release of NPM1c from its targets with a consequent decrease in the expression of HOX/MEIS. NPM1c is particularly enriched at active chromatin sites, where MLL1 and RNA Polymerase II (Pol II) are also located (11). Specific degradation of NPM1c leads to reduced HOX/MEIS transcription within

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Table 1. Features of *NPM1*-mutated AML.

Definition and epidemiology	• AML with cytoplasmic nucleophosmin (NPM1c+) is synonym of *NPM1*-mutated AML • Approximately one third of adult AML (50%–60% AML with normal karyotype), less frequent in children (2%–8%) • Female predominance
Pathology and immunophenotype	• Bone marrow is usually markedly hypercellular. Reticulin fibers are not usually increased • Mostly myelomonocytic and monocytic differentiation (FAB M4 and M5), but all FAB categories are represented • Approximately 23% of cases show multilineage dysplasia • High WBC count is observed when concomitant *FLT3* or *RAS* mutations are present • Extramedullary involvement is common, especially skin (easily detectable by IHC)
Gene-expression profile	• GEP shows upregulation of HOX genes • No/low expression of CD34 (the rare CD34+ leukemic cells carry the *NPM1* mutation)
Response to therapy and prognosis	• Excellent response to induction chemotherapy but suboptimal response to hypomethylating agents • Relatively good outcome in the absence of *FLT3*-ITD. Comutations in *NPM1/N-RAS*, *NPM1/RAD21*, and *NPM1-FLT3-TKD* are also associated with good prognosis • Cases with *NPM1/FLT3-ITD*, *NPM1-WT1*, or *NPM1/FLT3-ITD/DNMT3A* comutations show poor outcome

Abbreviations: FAB, French–American–British; GEP, gene-expression profile; WBC, white blood cell count.

15 minutes, due to the loss of Pol II from these loci (11, 12). Notably, the NPM1 acidic domain (~aa 120–150) also plays an important role in recruiting NPM1c to chromatin (11).

Based on the above findings, we have hypothesized that NPM1c can promote leukemogenesis by acting at both the nuclear and cytoplasmic level, i.e., "killing two birds with one stone" (15). Haploinsufficiency for wild-type NPM1 at the nucleolar level (because of heterozygosity of *NPM1* mutation and aberrant localization of the NPM1 native protein in the cytoplasm through the formation of heterodimers with the NPM1 mutant) may also play a role in the mechanism of leukemogenesis.

Frameshift indel mutations (such as *NPM1* mutation A) are also responsible for the unique immunologic features of *NPM1*-mutated AML cells. In fact, these somatic mutations can generate tumor-specific neoepitopes, which, after proteasomal degradation, processing in the endoplasmic reticulum, and loading as neoantigens onto the cell's major histocompatibility complex, are recognized by a patient's autologous CD4+ and CD8+ T cells, giving rise to an immune response. The ectopic cytoplasmic location of mutated NPM1 protein may enhance its processing by the human leukocyte antigen (HLA) class 1 pathway, leading to efficient antigen presentation. We proposed for the first time that C-terminus peptides of the NPM1 mutants can bind HLA class I molecules (16). Other investigators have subsequently confirmed this original report (17).

A number of observations suggest that neoantigens generated from mutated NPM1 protein are potential targets for immunotherapy. First, *NPM1* mutations are very frequent and specific for AML, are driver genetic events that are not associated with clonal hematopoiesis, and are stable at relapse (2). Second, NPM1 neoepitopes are not subject to central immune tolerance and are not expressed in normal tissues. Third, despite the large number of mutation sequences reported, two of these (types A and B) account for >80% of patients. Fourth, the C-terminal sequence of type A mutated NPM1 protein generates a strong immune response, including specific antibodies, in animal models. Finally, T cells, which can generate a response to peptides from mutated NPM1, can be observed in patients treated for *NPM1*-mutated AML (18, 19), including those in molecular complete remission (20), and it has been speculated that this might contribute to the favorable outcomes of this AML subtype (21). Therefore, cellular therapies (22, 23) and cancer vaccines that target mutated NPM1 epitopes are of great interest.

The distinction of *NPM1*-mutated AML from other AML genotypes can sometimes be difficult (24), and the percentage of blasts required for its diagnosis remains controversial (25). Immunohistochemistry techniques for detecting cytoplasmic NPM1 (26) can provide complementary information to molecular techniques. Determining the mutation status of NPM1 is essential for risk stratification in the European LeukaemiaNet (ELN) guidelines (27); moreover, this identifies patients for monitoring of measurable residual disease (MRD) by qRT-PCR (28, 29), which can further refine risk stratification and guide therapeutic decisions after remission. Here, we review the criteria for the appropriate diagnosis, risk stratification, and molecular monitoring of *NPM1*-mutated AML.

DETECTION TECHNIQUES OF *NPM1*-MUTATED AML

NPM1 mutations can be identified by molecular assays or by surrogate techniques, including IHC. These methods are complementary and allow a flexible approach to the diagnosis of *NPM1*-mutated AML which is critical for implementing the use of the ICC and WHO classifications worldwide.

Qualitative Detection of *NPM1* Mutations by Molecular Techniques

NPM1 mutations occur in about one-third of adult AML patients (1). Conversely, they are uncommon in childhood (about 8% of cases; ref. 30), where they are usually non-type A (31). *NPM1* mutation A (a duplication of TCTG at

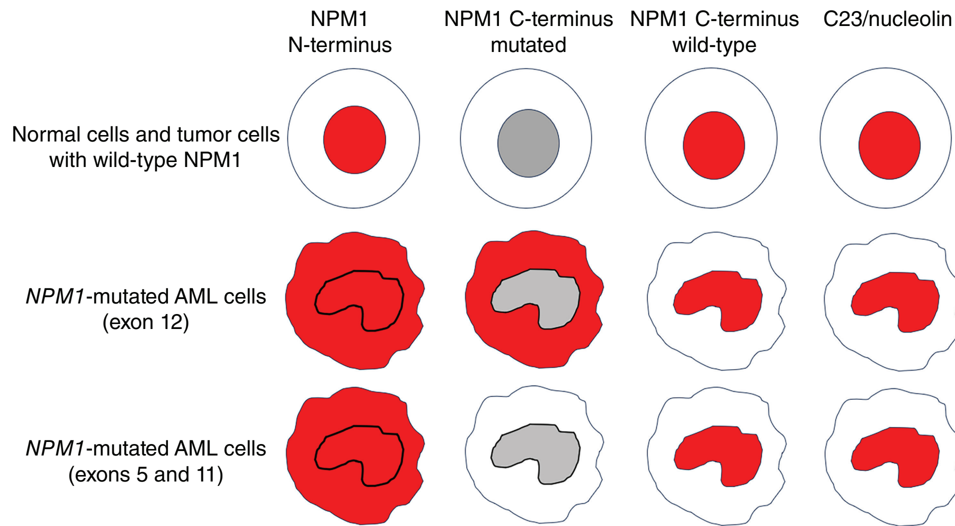

Figure 1. Representative examples of subcellular (nuclear and/or cytoplasmic) expression of NPM1 and nucleolin (red staining) in normal tissues, NPM1 wild-type tumors, and NPM1-mutated AML.

position 860–863 of the reference sequence) occurs in about 75%–80% of adult cases (1). Mutations B and D account for approximately 10% and 5% of cases, while other mutations are rare. More than 100 different types of *NPM1* mutations are now recognized. One distinguishing feature of *NPM1* mutations is that they do not drive clonal hematopoiesis. Thus, their presence at remission indicates active disease that can cause relapse and is associated with inferior outcomes. In this respect, *NPM1* mutations clearly differ from those involving the *DNMT3A*, *TET2*, and *ASXL1* genes that are associated with clonal hematopoiesis. These mutations can persist at remission, but they do not have prognostic value, simply reflecting the reestablishment of a preleukemic state following therapy for AML that does not require further treatment.

Qualitative assays for *NPM1* mutations are most commonly based on genomic DNA as a substrate and use PCR followed by fragment length analysis to detect the insertion, although assays based on melting curve analysis and qRT-PCR are also available (32–35). In general, PCR fragment analysis methods are preferred because they can detect all insertions within the PCR amplicon regardless of the mutation sequence, and they are simple and rapid, affording a sensitivity of ~5%, which is adequate in almost all cases (32). The exception to this is myeloid sarcoma, where the establishment of submicroscopic bone marrow (BM) involvement requires a more sensitive technique such as qRT-PCR (32).

Qualitative assays are best applied to fresh BM or peripheral blood (PB) leukemic cells (34) but plasma (36) is also suitable. Molecular detection of *NPM1* mutations in paraffin-embedded trephines is unreliable due to the denaturing effect of decalcifying agents on nucleic acids. However, DNA extracted from paraffin-embedded tissue biopsies in cases of myeloid sarcoma is usually adequate for PCR fragment analysis (33).

Following the identification of an *NPM1* mutation, for patients where MRD monitoring is planned, it is essential to establish the mutation sequence and to store both DNA and RNA to allow the determination of baseline transcript levels to permit comparison with post-remission samples (discussed further below; ref. 37). Next-generation sequencing (NGS) is increasingly used in the diagnosis of AML (27) and has the advantages of providing the insertion sequence, the ability to detect mutations outside exon 12 if an appropriate panel is used, and to identify comutations that may also have a prognostic impact. A recent study investigated the interlaboratory concordance in identifying driver mutations in AML (*DNMT3A*, *FLT3*, *IDH1*, *IDH2*, *NPM1*, *TET2*, *TP53*, and *WT1*) using different NGS platforms and found concordance >95%, with perfect agreement for *NPM1* mutations (38). Given the increasing need to rapidly molecularly stratify patients prior to treatment initiation, it is likely that rapid PCR-based methods for *NPM1* and other mutations that could influence first-line treatment choice will play a role in the foreseeable future.

IHC Detection of Cytoplasmic NPM1

The aberrant export of NPM1 (1) to the cytoplasm of leukemic cells (also referred to as NPM1c$^+$) is the result of the mutation-induced changes at the C-terminus of NPM1, i.e., loss of one or two of the tryptophans at positions 288 and 290 and addition of a *de novo* NES motif (3, 39) that enhances the interaction with the nuclear exporter XPO1. New C-terminal NES motifs of different strength are inserted to further tune the nuclear-cytoplasmic shuttling of the mutant (3).

IHC detection of NPM1c$^+$ is a simple, low-cost, very sensitive, and specific alternative assay to diagnose *NPM1*-mutated AML (26) that can serve as a surrogate to molecular assays. Interestingly, IHC also allows the study of the genetic lesion at the protein level in tissue sections and may provide information on the topographical distribution of the *NPM1*-mutated leukemic cells (e.g., in the paratrabecular area or close to the vessels). The pattern of reactivity of monoclonal antibodies directed against different domains of NPM1 and C23/nucleolin is shown in Fig. 1.

IHC is critical for defining multilineage dysplasia, diagnosis of myeloid sarcoma (40, 41), recognition of cases with aplastic or necrotic BM, resulting in dry tap and identification of patients carrying *NPM1* mutations outside exon 12 that may be missed by standard molecular assays (see below; refs. 6, 42). This technique can also serve as a surrogate for the detection of *NPM1* mutations when molecular assays are not available, for example in some developing countries (43).

NPM1-mutated AML can be further characterized by molecular IHC using a monoclonal antibody specifically directed against the IDH1-R132H mutant protein (44), as *NPM1* mutations associate more frequently with IDH1-R132H than with other amino acid changes (i.e., IDH1-R132C, R132G, R132S; ref. 44).

Detection of *NPM1*-Mutated Proteins by Flow Cytometry

Diagnosis of *NPM1*-mutated AML by flow cytometry is based either on the detection of cytoplasmic NPM1 (45) or a particular phenotype. A recent study highlighted unique immunophenotypic patterns associated with *NPM1*-mutated AML, including the presence of (i) immature $CD34^{lo}/HLA\text{-}DR^{lo}/CD15^{+}/CD7^{+}$ AML cells and/or, (ii) neutrophil lineage AML cells displaying low CD34, CD71, and CD64, while upregulating CD105 and/or, (iii) monocytic leukemia cells with $CD34^{lo}$ and asynchronous ($CD300e^{+}CD14^{-}$; $CD35^{+}CD14^{-}$) phenotypes (46). Moreover, the *NPM1*-mutated/*FLT3*-ITD genotype was closely associated with a $CD7^{hi}$ $CD38^{lo}$ phenotype on immature leukemia cells and/or $CD117^{het}$ and $CD123^{hi}$ expression on neutrophil lineage-committed AML cells (46).

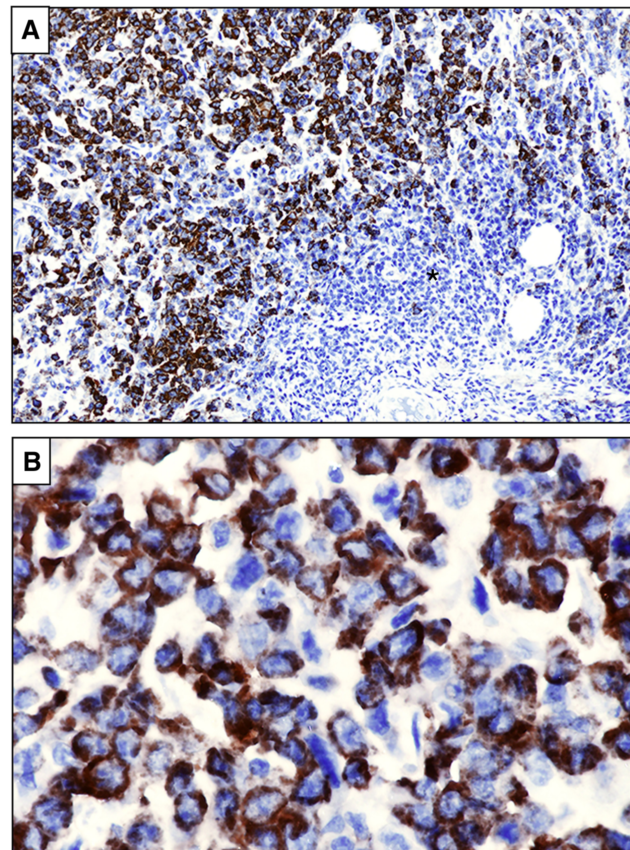

Figure 2. NPM1-mutated myeloid sarcoma (lymph node). **A,** Partial infiltration of the lymph node (asterisk) by leukemic cells showing aberrant cytoplasmic expression of nucleophosmin (brown; immunoperoxidase; hematoxylin counterstaining; × 100. **B,** The same field showing NPM1 cytoplasmic positive tumor cells at higher magnification (immunoperoxidase; hematoxylin counterstaining; × 400).

DIAGNOSTIC PITFALLS IN *NPM1*-MUTATED AML

NPM1-mutated AML can be diagnosed when *NPM1* mutation and/or aberrant cytoplasmic expression of NPM1 are detected in a patient meeting other criteria for diagnosis of AML. Other features that may help to predict *NPM1*-mutated AML pending molecular confirmation include occurrence in a middle-age or older patient, relatively preserved number of platelets despite high white blood cell (WBC) count, blasts with myelomonocytic/monocytic (M4–M5) differentiation, multilineage involvement, cup-like morphology, CD34 and HLA-DR negativity, normal cytogenetics, and skin involvement (24, 47). Patients comutated for *NPM1* and *FLT3*-ITD or *RAS* frequently present with hyperleukocytosis (40) and may show a starry sky pattern (48). Conversely, *NPM1*-mutated AML without *FLT3* or *RAS* mutations usually presents with low–normal WBC count (40). The BM in *NPM1*-mutated AML is characteristically hypercellular but reticulin fibers are usually not increased. Pitfalls in the diagnosis of *NPM1*-mutated AML are discussed below.

AML with Multilineage Dysplasia

About 23% of *NPM1*-mutated AML display multilineage dysplasia (ref. 49; i.e., dysplasia ≥50% of cells, in at least two BM cell lineages). These cases may be misdiagnosed as myelodysplasia (MDS) or AML/MDS. Demonstration of *NPM1* mutation establishes the diagnosis because the genetic lesion supersedes morphology in importance (4, 40). IHC confirms the multilineage involvement by demonstrating aberrant cytoplasmic expression of nucleophosmin in precursors of erythroid and myeloid lineages and even in mature megakaryocytes.

Myeloid Sarcoma

Extramedullary involvement is not infrequent in *NPM1*-mutated AML, with the skin being one of the most commonly involved anatomic sites (24, 47, 50), especially in cases with monocytic or myelomonocytic features. As compared with *NPM1*-mutated AML, *NPM1*-mutated myeloid sarcoma appears to show differences in genomic landscape, including a higher frequency of cytogenetic abnormalities and mutations affecting epigenetic modifiers such as *ASXL1*, and a lower frequency of mutations affecting *PTPN11, DNMT3A*, and *IDH1* (51). *NPM1*-mutated myeloid sarcoma also has poorer overall survival than *NPM1*-mutated AML (51).

IHC detection of cytoplasmic NPM1 is more reliable than molecular assays in establishing the diagnosis of *NPM1*-mutated myeloid sarcoma (refs. 24, 41; Fig. 2A and B), particularly when only a small biopsy sample is available for analysis (e.g., punch biopsy). Whether extramedullary involvement represents a poor prognostic factor in *NPM1*-mutated AML remains controversial. In a large study on >3,000 patients

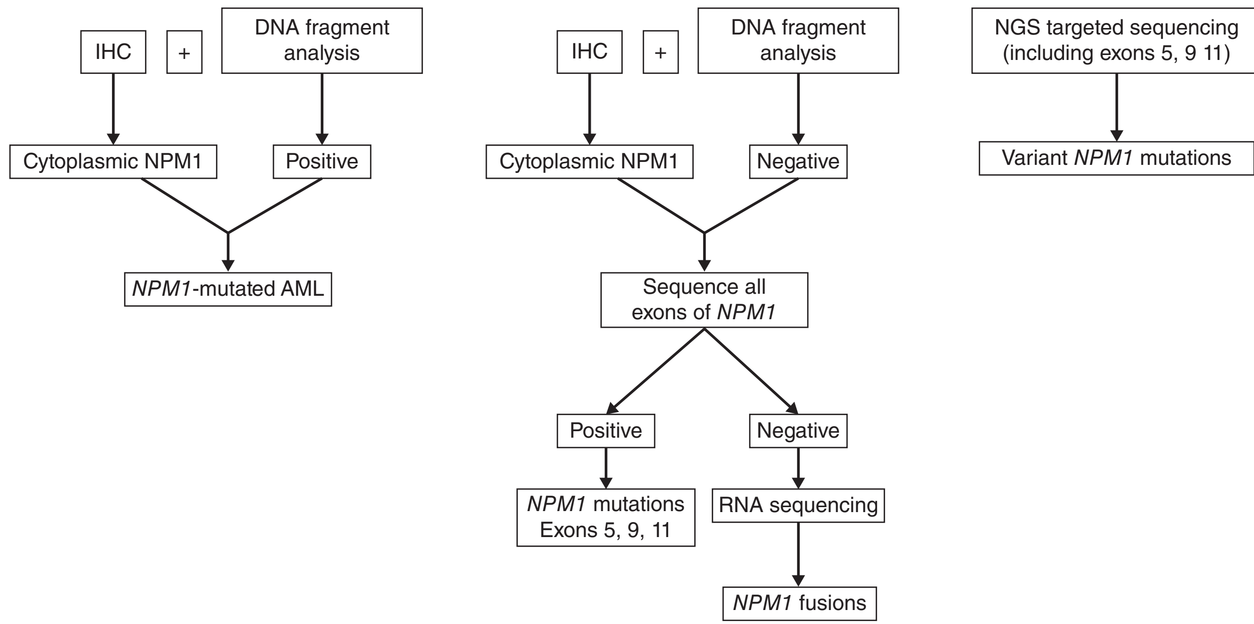

Figure 3. IHC and molecular procedure for recognizing NPM1 mutations occurring outside exon 12 and NPM1-containing fusion proteins.

with AML, extramedullary disease did not emerge as an independent prognostic factor (52) but mutational status was not considered. Molecular discordance between myeloid sarcomas and concurrent BM (defined as the occurrence of different mutations in either sample) was associated with worse overall survival, probably due to increased clonal heterogeneity and resistance to therapy (53).

Identification of *NPM1* Mutation Outside Exon 12

NPM1 mutations almost exclusively affect exon 12 (1). Mutations involving exons 9 (3), 11 (3), and 5 (6, 42) may rarely occur. Independently of the exon involved, all *NPM1* mutations lead to similar changes at the C-terminus that result in the increased export of NPM1 mutant protein and its accumulation in the cytoplasm of leukemic cells (3). For this reason, IHC is an excellent method for detecting *NPM1* mutations occurring outside exon 12 (26). If IHC and molecular assays are used in combination, a discrepancy between the two techniques (i.e., detection of cytoplasmic NPM1 in the absence of mutation at exon 12) should prompt analysis of the entire *NPM1* coding sequence, to identify mutations in other exons (Fig. 3). Aberrant cytoplasmic localization of exon 11 and exon 5 NPM1 mutants is identified by antibodies directed against the N-terminus of NPM1 but not by antibodies specific for the NPM1 mutant (Fig. 1). In fact, exon 11 and exon 5 mutations result in the translation of either truncated proteins or longer mutants retaining the same C-terminus sequence as the NPM1 wild-type (6). Although NGS can identify mutations occurring in any exon, many commercially available NGS panels only target exon 12; therefore, custom panels may be required for the detection of other mutations (Fig. 3).

Inability to recognize exon 11 and 5 mutations may lead to the incorrect assignment of these cases to the ELN intermediate-risk (*NPM1* wild-type without *FLT3*-ITD) rather than to the favorable-risk group (*NPM1*-mutated without *FLT3*-ITD). Because of the low number of cases analyzed, it remains unclear whether these patients have the same outcome as the typical patients with exon 12 *NPM1* mutations. So far there are little data regarding MRD monitoring for *NPM1* mutations outside exon 12, which requires patient-specific qRT-PCR assays.

AML with Concomitant *NPM1* Mutations and BCR::ABL

The association of *NPM1* mutations with *BCR::ABL1* has been rarely reported in AML (40, 54, 55). These cases should be classified as *NPM1*-mutated AML, annotating the presence of *BCR::ABL1*. This is also supported by their CD34 negativity, which is unusual in *BCR::ABL1* AML. *NPM1*-mutated AML without *FLT3*-ITD has a relatively good outcome (27), whereas AML with *BCR::ABL1* is a high-risk leukemia. ELN does not provide prognostic information on cases carrying both *NPM1* mutations and *BCR::ABL1*. These cases immunophenotypically and clinically behave more like an *NPM1*-mutated AML than AML with *BCR::ABL1*, but further studies are warranted to clarify this issue.

Therapy-Related *NPM1*-Mutated AML

NPM1 mutations are characteristically detected in AML of *de novo* origin (1). About, 15% of cases of therapy-related AML that occur after previous cytotoxic chemotherapy and/or radiotherapy harbor *NPM1* mutations (56, 57). These cases have many overlapping biological and clinical features with *de novo* *NPM1*-mutated AML. In fact, they consistently show normal cytogenetics (56), *DNMT3A* (58–60), and *TET2* (60) mutations, cytoplasmic NPM1, and a gene-expression profile characterized by upregulation of HOX genes and downregulation of CD34 (60). Moreover, the rate of *TP53* (61) and *PPM1D* mutations (62) that are responsible for chemoradiotherapy-driven selection is much lower in therapy-related *NPM1*-mutated

AML (3% and 4%, respectively; ref. 60) than in therapy-related AML with wild-type *NPM1* (25% and up to 20%, respectively; refs. 61, 63). Finally, the survival of therapy-related and *de novo NPM1*-mutated AML was similar but differed significantly from that of therapy-related AML with wild-type *NPM1* (60, 64). Collectively, these findings clearly indicate that the leukemic mechanism underlying "therapy-related" *NPM1*-mutated AML differs from that of other therapy-related AMLs and most likely represents a *de novo* leukemia with a coincidental history of prior therapy (60). Based on these findings, therapy-related *NPM1*-mutated AML is now regarded in both the 2022 ICC (4) and WHO-5 (5) classifications as *NPM1*-mutated AML (with the addition of "therapy-related" or "post-cytotoxic therapy" as qualifier). Therapy-related *NPM1*-mutated AML without *FLT3*-ITD should be assigned to the ELN favorable group and transplant decisions guided by MRD assessment, as in patients with typical *de novo NPM1*-mutated AML (27).

Percentage of Blasts Defining *NPM1*-Mutated AML

According to the 5th edition of WHO (5), the diagnosis of *NPM1*-mutated AML can be made irrespective of the percentage of blasts while the 2022 ICC (4) still requires ≥10% blasts. Thus, the question of how an *NPM1*-mutated myeloid neoplasm with <10% blasts should be diagnosed still remains open. In the past, such cases were usually classified as *NPM1*-mutated myelodysplastic syndrome (MDS) or chronic myelomonocytic leukemia (CMML; refs. 65, 66) with mutated *NPM1*. However, they generally resemble more closely *NPM1*-mutated AML than MDS or CMML with wild-type *NPM1*. In fact, they usually show a normal karyotype and lack the typical mutations (*ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1*, and *ZRSR2*; refs. 65, 66) that define MDS or AML with myelodysplasia-related mutations (4). Moreover, they show CD34 negativity, a tendency to evolve rapidly to AML (67) especially when the *NPM1* mutation allelic burden is high (65, 66), and good response to chemotherapy but suboptimal response to hypomethylating agents (65, 66, 68). Collectively, these findings strongly suggest that *NPM1*-mutated myeloid neoplasms with <10% blasts may represent *NPM1*-mutated AML diagnosed at an early stage and that the *NPM1* mutation defines AML irrespective of blast count (25). This concept is also supported by the IHC study of BM biopsies that show aggregates of NPM1 cytoplasmic blasts, usually outnumbering blast cells detectable by morphologic criteria alone (25). Thus, we suggest that these patients should be treated as typical *NPM1*-mutated AML.

PROGNOSTIC IMPACT OF *NPM1* MUTATIONS

Genetic-based risk stratification is part of routine work-up for the management of patients with AML. The median number of pathogenetic variants in newly diagnosed AML is four to five (69), with a frequent scenario being the stepwise accumulation of mutations beginning with those associated with clonal hematopoiesis (e.g., *DNMT3A* and *TET2*), followed by an AML-defining mutation (such as *NPM1*) with the acquisition of mutations in signaling pathway components (e.g., *FLT3, NRAS, KRAS*) as the final events in leukemogenesis (2). However, a large variety of mutational combinations, sometimes associated with secondary chromosomal abnormalities, may contribute to the genotype of *NPM1*-mutated AML (69).

Once the diagnosis of *NPM1*-mutated AML has been established, patients should be risk stratified according to the ELN 2022 guidelines (27). Patients with *NPM1* mutation in the absence of *FLT3*-ITD are assigned to the favorable-risk group. Those with *FLT3*-ITD (regardless of the allelic ratio) are assigned to the intermediate group because of the difficulty in reproducing measurement of the allelic ratio between laboratories and the recognition that MRD status plays an increasingly important role in risk stratification. Those with adverse karyotype are assigned to the high-risk group on the basis of a large meta-analysis (70). Accumulating evidence indicates that the outcome of *NPM1*-mutated AML may vary according to accompanying mutations other than *FLT3*-ITD. Very large studies of uniformly treated patients are required to confidently assign risk associations to mutational subgroups, and current data should be regarded as provisional. In a study including 435 patients with *NPM1* mutation enrolled in sequential intensive treatment protocols (69), patients with both *FLT3*-ITD and *DNMT3A* comutations (n = 93) showed a particularly poor outcome, while those with either *NRAS* codon 12 (n = 69) or *RAD21* (n = 33) mutations appeared to show improved overall survival. A similar study involving 297 *NPM1*-mutated patients identified a poor prognosis in those with *WT1* comutation (71). An analysis using combined data from multiple cooperative group studies (72), including 1,093 patients with *NPM1* mutation, demonstrated complex interactions between comutations but again identified a very poor outcome in those with both *FLT3*-ITD and *DNMT3A* comutations. NRAS, KRAS, PTPN11, and RAD21 mutations were associated with favorable prognosis in the absence of FLT3-ITD and IDH1.

Both the ICC and WHO classifications of AML prioritize *NPM1* mutation status above myelodysplasia-related (MR; "secondary type") mutations (*SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR*, and *STAG2*) in cases where these cooccur (73, 74). The suggestion of the ELN panel (27) that myelodysplasia-related mutations should not overrule the favorable impact of a cooccurring *NPM1* mutation is further supported by a recent study including 936 patients with *NPM1*-mutated AML in which myelodysplasia-related mutations did not affect outcomes in this cohort (75). *NPM1*-mutated AML cases with cooccurring myelodysplasia-related mutations were significantly older and showed lower levels of WBC and platelets than cases without cooccurring mutations (75); however, there were no differences in rates of complete response (CR), relapse-free survival (RFS), or overall survival (OS; ref. 75). Therefore, these patients should still be considered ELN favorable risk and treated accordingly (75).

To date, the only genotype consistently associated with poor outcome (76) not fully captured in the ELN scheme is the combination of *NPM1*, *FLT3*-ITD, and *DNMT3A* mutations. However, it remains unclear whether or how this finding should influence treatment.

Decisions regarding postremission therapy are usually guided by the ELN scheme. However, it is proposed that assessment of MRD should be used to reclassify patients; thus favorable-risk patients with a poor MRD response could be considered candidates for allogeneic hematopoietic stem cell transplantation (allo-HSCT), whereas intermediate-risk patients with a good MRD response could potentially avoid this procedure.

Although the limited data published to date support this approach, the selection of patients for allo-HSCT remains somewhat controversial. There is currently no direct evidence that patients with favorable-risk *NPM1* mutated AML testing MRD-positive benefit from allo-HSCT in CR1, although results from trial protocols where these patients were directed to transplant have been very encouraging (77). For intermediate-risk patients, a post-hoc analysis from the ALFA 0702 study (78) suggested that only patients with an unfavorable MRD response benefited from allo-HSCT. More recently, this has been further supported by data from the HOVON (79) and GIMEMA (80) groups where patients in the intermediate-risk group (regardless of baseline *NPM1* status) were allocated to either autograft or allo-HSCT on the basis of MRD results after the second cycle of intensive chemotherapy, resulting in identical outcomes between the MRD-positive and negative groups. In the context of patients with *NPM1* and *FLT3*-ITD mutations, this remains controversial because of earlier studies showing a benefit of transplant, especially in patients with a high allelic ratio (81). These studies did not incorporate MRD assessment, and therefore the benefit of allo-HSCT for patients with *NPM1* and *FLT3*-ITD mutations who achieve MRD negativity remains uncertain, although protocols wherein only MRD-positive patients were directed to allo-HSCT have shown excellent results in this group (77). Similarly, for patients with other high-risk genotypes including "triple-hit" there is a lack of data regarding the benefit of transplant in patients achieving MRD negativity; however, in one study these patients had a relatively favorable outcome (3-year OS 70%; ref. 29).

In summary, although further data are clearly needed, the current ELN risk stratification scheme, with appropriate reclassification between the favorable and intermediate-risk groups based on MRD status, should remain the basis for decisions regarding post-remission therapy.

Table 2. Features of MRD marker mutations in acute myeloid leukemia.

Feature	*DTA*[a]	*NPM1*	FLT3-ITD
Clonal hierarchy	Preleukemic	Leukemia-initiating	Usually subclone
High frequency	Yes	Yes	Yes
AML specificity	No	Yes	No
Distinct GEP	No	Yes	No
Clonal hematopoiesis	Yes	No	No
Clearance post-morphologic CR	No	Yes	Yes
Prediction of relapse	No	Yes	No
Stability at relapse	Yes	Yes	No
Sample source	gDNA	Usually cDNA	gDNA

Abbreviations: GEP, gene-expression profile; CR, complete remission; gDNA, genomic DNA.

[a]*DTA: DNMT3A, TET2, ASLX1* mutations.

MOLECULAR MRD ASSESSMENT IN *NPM1*-MUTATED AML

NPM1 mutations are an ideal target for monitoring subclinical levels of leukemia (i.e., MRD), because they are common, AML-specific, not expressed in normal tissues and absent in the preleukemic state (e.g., clonal hematopoiesis; Table 2). Moreover, they correlate with therapeutic response, recur at disease relapse, and rising levels of MRD (called MRD relapse) reliably predict clinical relapse within a period of weeks/months, allowing time for preemptive intervention (Table 2). MRD evaluation, including that of *NPM1* mutant transcripts, is now routinely recommended by the European LeukemiaNet (27) to evaluate the molecular response to treatment in AML, while associated preleukemic mutations "should be excluded from MRD analysis." This approach may also be used to guide MRD-directed therapy with novel, less toxic drugs that have been approved in AML. Finally, the approval of NPM1 MRD as a regulatory endpoint is expected to markedly change the clinical trial landscape, such as biomarker-driven adaptive design.

NPM1 mutant transcripts are almost always insertions in a small hotspot, making them ideal targets for qRT-PCR detection using RNA as a substrate, and this is the currently recommended method (37). This method provides very high sensitivity, particularly when BM samples are used (sensitivity is approximately one-log lower when using PB). In a recent study, a significant proportion of false-positive results in the *NPM1* wild-type sample was reported among 29 laboratories (82). False-positive results may result in erroneous clinical decisions, e.g., planning unnecessary additional chemotherapy and/or allotransplant with the consequent risk of morbidity and mortality, underlying the need for extensive validation, rigorous negative controls, and external quality assurance when these assays are used to inform clinical decision-making.

There is increasing interest in the use of digital droplet PCR (83–85) and NGS-based ultra-deep sequencing (86, 87). Advantages of digital droplet PCR include high sensitivity (when using RNA as input) and the ability to quantify rare mutations (i.e., non-type A, B, or D) without the need for a standard curve (88). NGS-based strategies can detect all types of *NPM1* mutants, but they currently lack standardization and currently have lower sensitivity, particularly those using genomic DNA as a substrate; therefore, they are currently recommended only in the context of clinical trials (37).

Molecular assessment of MRD in *NPM1*-mutated AML has a number of different clinical applications: (i) MRD status at post-induction time points is strongly predictive of relapse and OS (29, 80, 86, 89) and therefore can be used to refine risk assessment within the current ELN scheme, allowing more rational selection of patients for allo-HSCT; (ii) pretransplant MRD assessment is highly predictive of outcome, and may inform peritransplant management; (iii) sequential MRD assessment allows the detection of MRD relapse, which predicts impending clinical relapse (90, 91) but provides a window period allowing for preemptive intervention (92–94); (iv) it can be used to define a new response category of CR_{MRD-} for use in both routine practice and clinical studies; and (v) it can serve as surrogate endpoint to accelerate drug testing and approval (27). A possible approach to the incorporation

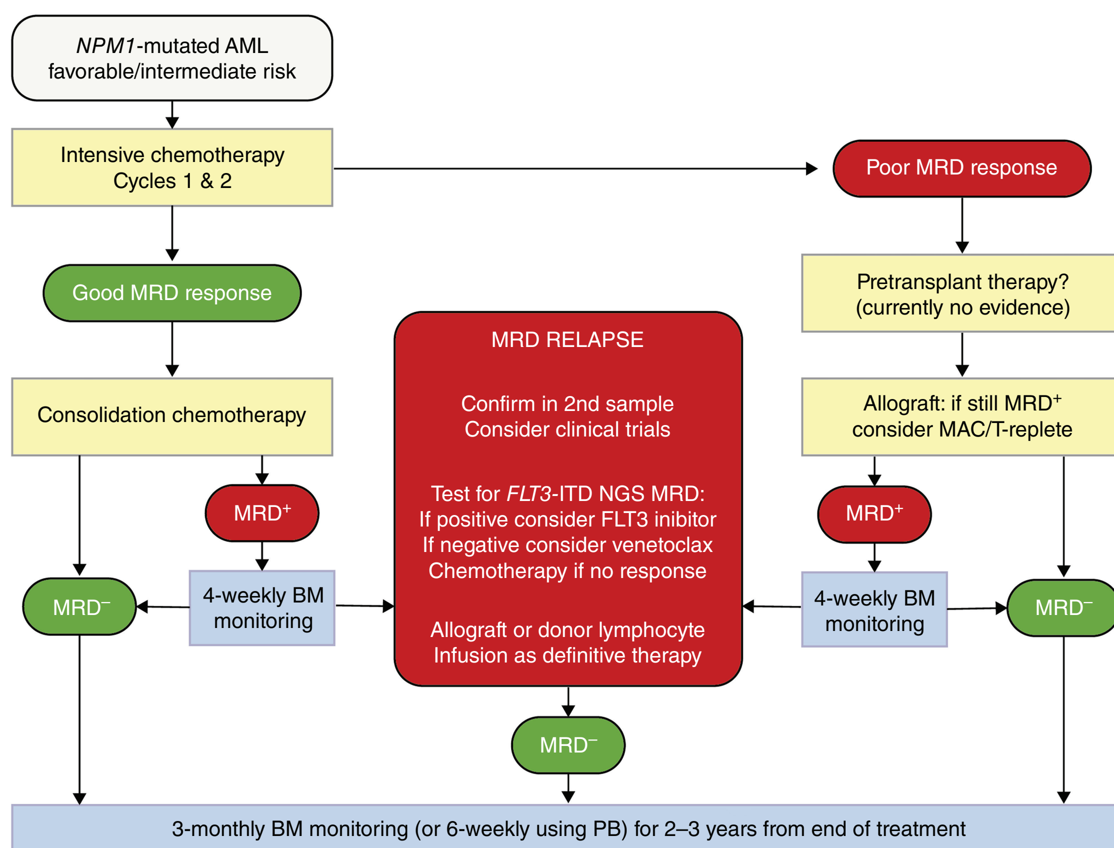

Figure 4. Possible approach to the incorporation of MRD into treatment algorithms for patients with *NPM1*-mutated AML.

of MRD into treatment algorithms for patients with *NPM1*-mutated AML is shown in Fig. 4.

Multiparameter flow cytometry (MFC) has a limited role in monitoring MRD in *NPM1*-mutated AML because leukemia-associated immunophenotypes (LAIP) are not entirely specific. Moreover, persistent clonal hematopoiesis following eradication of the *NPM1* mutation may lead to spurious detection of MRD by MFC.

NPM1 MRD Assessment at Postinduction Time Points

Several large prospective clinical trials have demonstrated the prognostic effect of MRD measured by qRT-PCR at early time points, although precise cutoffs defining high-risk groups differ between studies. In the German AMLSG 0704 study (90), patients achieving MRD negativity in the BM after cycle 2 had a 4-year cumulative incidence of relapse (CIR) 6% versus 53% and 4-year OS 90% versus 51%. The subsequent AMLSG 0909 study (95) reported 4-year CIR for BM MRD negativity after cycle 2 of 25% versus 38% ($n = 370$) and also showed the prognostic impact of PB MRD negativity (4-year CIR 18% vs. 53%, $n = 341$). This study also identified a 3-log reduction in both PB and BM as prognostically important. The French ALFA 0702 study (78) identified a >4-log reduction in PB MRD after cycle 1 as most predictive of outcome (3 year CIR 21% vs. 66%, 3y OS 92% vs. 41%). Finally, the UK NCRI AML17 study (29) reported that the PB MRD status after cycle 2 had the strongest prognostic impact (3-year CIR 28% vs. 83%, 3-year OS 77% vs. 25%). Thus, although clearly of prognostic value, further work is needed to identify a unified threshold most predictive of poor outcomes.

Evaluation of *NPM1* MRD has a strong prognostic value even in patients treated with venetoclax-based nonintensive therapies. In particular, patients achieving BM MRD negativity by the end of cycle 4 had a 2-year OS of 84% compared with 46% if MRD-positive. On multivariable analyses, MRD negativity was the strongest prognostic factor (96).

NPM1-mutated AML shows high expression of CD33 and a good response to gemtuzumab ozogamicin (GO). In the ALFA-0701 study, patients with *NPM1* mutation treated with GO showed deeper MRD responses at multiple time points compared with those not receiving GO (97). The same findings were observed in the AMLSG 09-09 trial, which also showed a lower 4-year CIR and higher RFS in patients who received GO (95).

NPM1 MRD Assessment At End of Treatment

Although MRD positivity at the end of treatment is associated with increased relapse risk, a proportion of these patients will remain in long-term remission, some even converting to CR_{MRD^-} without further therapy. The molecular detection of persistent *NPM1* mutation is notably agnostic to the cell type(s) carrying the mutant allele. Thus, at least in some patients with persistently low expression of *NPM1* mutant transcripts, the residual *NPM1*-mutated cells may include at least a subset that

are incapable of driving relapse. This is consistent with the observation that IHC of posttreatment MRD-positive samples shows terminally differentiated monocytic elements and mature megakaryocytes with cytoplasmic expression of NPM1. These differentiated cell types contribute to the measurement of mutant *NPM1* transcripts but may be irrelevant to the risk of relapse. Therefore, MRD positivity at the end of treatment should not automatically trigger further therapy.

In a UK–Australian study (98), 42% of patients with detectable *NPM1*-mutant transcripts at the end of treatment remained progression free at one year, and of these, 30% spontaneously achieved molecular negativity. Risk factors for progression included baseline *FLT3*-ITD and <4.4-log MRD reduction from diagnosis; 93% of patients with both of these factors relapsed and died within one year. The AMLSG 0704 study identified a threshold of 200 copies/10^4 *ABL* with 100% specificity for relapse; however, this threshold appeared less specific in the AMLSG 0909 study (4-year CIR 67%; refs. 90, 95). Again, efforts to define a unified threshold for intervention are now required. Here, a major concern is the danger of overtreating patients who are not destined to relapse. However, MRD positivity should certainly prompt closer monitoring (e.g., every 4–6 weeks) allowing intervention in the case of MRD relapse.

NPM1 MRD Assessment Before Transplant

Although many studies show that pretransplant MRD is strongly associated with poor outcome (99), few studies have investigated this specifically in *NPM1*-mutated AML. Here, the relationship between MRD and outcome appears more complex, being affected by both the level of MRD and *FLT3* mutation status. In the NCRI AML17 study (n = 107; ref. 100), a threshold of 200 copies/10^5 *ABL* in the PB or 1,000 copies in the BM defined a group with poor outcome (3-year OS 13%). The same BM threshold was identified in a German study (n = 67, 5-year OS 40% vs. 89%; ref. 101). In AML17, patients who were MRD-positive below these levels and who were *FLT3*-ITD-negative at baseline had the same outcome as those testing MRD-negative (2-year OS 82%), whereas those who had *FLT3*-ITD at baseline had poor outcomes (2-year OS 17%). A subsequent study using a sensitive NGS-based *FLT3*-ITD MRD assay (102) could further stratify patient outcome: 2-year OS for patients testing MRD$^-$, MRD$^+$ for *NPM1* only and MRD$^+$ for *NPM1* and *FLT3*-ITD in the pretransplant sample was 82%, 68%, and 25%. The US “Pre-Measure” study (103) also showed poor outcomes in patients with detectable pretransplant *NPM1* MRD (3-year CIR 63% vs. 22%, 3-year OS 35% vs. 66%). This study used a genomic DNA-based assay with less sensitive PB samples, supporting the concept of a threshold effect, and confirmed the additional prognostic impact of *FLT3* MRD status (3-year CIR 64% vs. 75% and 3-year OS 40% vs. 25% for patients testing MRD$^+$ for *NPM1* only and MRD$^+$ for *NPM1* and *FLT3*).

Whether myeloablative conditioning can reduce relapse risk in *NPM1* MRD-positive patients remains controversial (100, 104, 105). In one study, myeloablative HLA-haploidentical transplantation with regulatory and conventional T cell–adoptive therapy (106) was shown to dramatically reduce the CIR. Studies evaluating the use of hypomethylating agents plus venetoclax during salvage therapy are also of interest (107), especially because *NPM1*-mutated AML is particularly sensitive to this combination therapy.

Sequential Monitoring for MRD Relapse

Regardless of first-line treatment approach and MRD status, all patients remain at a nontrivial risk of relapse for the first 2–3 years after therapy. Sequential MRD monitoring can be used to reliably identify patients destined to relapse (29) for preemptive intervention. However, overtreatment of patients who will not relapse remains a primary concern; therefore, stringent criteria for diagnosing MRD relapse have been proposed (37). These require two consecutive samples confirming conversion from MRD$^-$ to MRD$^+$ for patients who have previously tested MRD$^-$ in >1 technically adequate sample. Otherwise, a 1-log increase, confirmed in a second sample, is required.

The optimal treatment for patients with MRD relapse remains undefined. Although standard salvage chemotherapy appears effective, with MRD negativity achieved in ~60% (100), targeted therapies may provide a less toxic alternative without requiring hospital admission. One of the first examples of MRD-directed therapy (excluding acute promyelocytic leukemia) was the use of 5-azacytidine to treat patients with *NPM1*-mutated AML in molecular relapse (108) or experiencing a molecular relapse following allo-HSCT (phase II RELAZA2 study; ref. 92). In the latter study, 60% of 53 patients were *NPM1*-mutated, and 31 (58%) patients had an MRD response, including 19 (36%) who achieved MRD negativity (92). Notably, 58% of patients remained relapse free at 6 months from therapy initiation, and the 2-year RFS was 46% (92).

Venetoclax-based regimens have also been applied as MRD-directed therapy, especially in *NPM1*-mutated AML, which is recognized as a predictive biomarker of response to venetoclax (109). Venetoclax with azacytidine or low-dose cytarabine produced rates of molecular negativity of 80% to 90% in small retrospective studies (93, 94). Based on these findings, the off-label combination of venetoclax plus azacitidine as a bridge-to-transplant strategy has been retrospectively evaluated in *NPM1*-mutated MRD-positive fit AML patients (94). After a median number of two cycles (range, 1–4), 9 of 11 patients (81.8%) achieved MRD-negative CR. All 11 patients proceeded to allo-HSCT; 10 of 11 patients are alive, with 9 of 10 being in MRD-negative status.

For patients with baseline *FLT3* mutation, a retrospective study (110) showed a molecular response to FLT3 inhibitors (>1-log reduction in MRD) in 60% and MRD negativity in 45%. *FLT3*-ITD NGS MRD could identify patients more likely to respond. For patients who have MRD relapse after allo-HSCT, donor lymphocyte infusion provides an additional option for eradicating MRD, with evidence of immune response against epitopes derived from mutated NPM1 protein (20).

MRD relapse may provide an extremely useful setting for early-phase evaluation of novel targeted agents. Historically, early-phase studies have usually been performed in patients with frank relapse. A major potential advantage of the MRD relapse setting includes usually normal baseline hematopoietic function (removing the difficulty of deconvoluting disease versus treatment-related hematologic toxicity and infections) and lower baseline disease burden (reducing the chance for clonal evolution or other adaptive therapy resistance). Additionally, sequential MRD monitoring provides a rapid,

objective, and highly sensitive method for efficacy evaluation. We anticipate that studies of new targeted agents and immunotherapies will be developed to exploit these advantages in the coming years.

Authors' Disclosures

B. Falini reports personal fees from European patent EP1944316B1 during the conduct of the study; in addition, B. Falini has a Eurepean patent, EP1944316B1, with royalties paid. R. Dillon reports grants and personal fees from AbbVie, grants from Amgen, Pfizer, and Jazz, and personal fees from Astellas outside the submitted work.

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