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How I diagnose and treat *NPM1*-mutated AML

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Mutations of the *nucleophosmin* (*NPM1*) gene, encoding for a nucleolar multifunctional protein, occur in approximately one-third of adult acute myeloid leukemia (AML). *NPM1*-mutated AML exhibits unique molecular, pathological, and clinical features, which led to its recognition as distinct entity in the 2017 World Health Organization (WHO) classification of myeloid neoplasms. Although WHO criteria for the diagnosis of *NPM1*-mutated AML are well established, its distinction from other AML entities may be difficult. Moreover, the percentage of blasts required to diagnose *NPM1*-mutated AML remains controversial. According to the European LeukemiaNet (ELN), determining the mutational status of *NPM1* (together with *FLT3*) is mandatory for accurate relapse-risk assessment. *NPM1* mutations are ideal targets for measurable residual disease (MRD) monitoring, since they are AML specific, frequent, very stable at relapse, and do not drive clonal hematopoiesis of undetermined significance. MRD monitoring by quantitative polymerase chain reaction of *NPM1*-mutant transcripts, possibly combined with ELN genetic-based risk stratification, can guide therapeutic decisions after remission. Furthermore, immunohistochemistry can be very useful in selected situations, such as diagnosis of *NPM1*-mutated myeloid sarcoma. Herein, we present 4 illustrative cases of *NPM1*-mutated AML that address important issues surrounding the biology, diagnosis, and therapy of this common form of leukemia. (*Blood*. 2021;137(5):589-599)

Introduction

Acute myeloid leukemia (AML) carrying mutations of nucleophosmin (*NPM1*), a gene encoding for a multifunctional nucleolar protein with chaperone and shuttling features,[1] accounts for ~30% of adult AML and exhibits distinctive molecular and clinicopathological features.[2,3] The aberrant cytoplasmic dislocation of mutant NPM1 is thought to play a key role in leukemogenesis.[4,5] *NPM1* mutations are infrequent (6.5%) in children,[6] peak at middle age, and tend to decrease in patients >70 years, although they can still be detected in ~20% of older AML patients.[7]

Following its discovery in 2005,[2] it has been a long road before *NPM1*-mutated AML was recognized by the World Health Organization (WHO) classification of hematopoietic neoplasms as provisional entity in 2008 and then as distinct entity in 2017.[8] According to the current WHO classification, *NPM1*-mutated AML (together with *CEBPA*-mutated AML) is 1 of 2 AML entities defined by a single gene mutation, although it is likely that this list will grow to include other genes. Because *NPM1*-mutated AML is relatively frequent, the WHO category of AML with recurrent genetic abnormalities has now approximately doubled in size, with ~60% to 65% of AML being recognizable by genetic assays. Moreover, simple and low-cost techniques, including immunohistochemistry (IHC) and basic molecular assays, are available for the detection of *NPM1* mutations, facilitating the diagnosis of *NPM1*-mutated AML worldwide,[9] which is a major scope of the WHO classification. We also believe that the recognition of *NPM1*-mutated AML as distinct entity encourages the design of genetic-based clinical trials. The main features of *NPM1*-mutated AML are summarized in Table 1.

According to the 2017 WHO classification,[8] diagnosis of *NPM1*-mutated AML requires ≥20% of blasts. Another critical diagnostic step is the identification of the exact type of *NPM1* mutation by sequencing.[11] This information is required to set up real-time quantitative polymerase chain reaction (RT-qPCR) for measurable residual disease (MRD) monitoring.[10] Detection of cytoplasmic NPM1 by IHC[12] may serve as surrogate for molecular diagnosis in cases of dry tap, bone marrow (BM) necrosis,[13] or myeloid sarcoma. Moreover, IHC enables detection of all clinically relevant *NPM1* mutations (even rare mutations occurring outside exon 12) and is feasible in developing countries not equipped for molecular studies.[9]

Criteria for distinguishing *NPM1*-mutated AML from other distinct AML entities are well defined.[8] *NPM1*-mutated AML must also be separated from 2 provisional entities of the 2017 WHO classification: AML with *BCR-ABL1* and AML with *RUNX1* mutations. When *NPM1* mutation and *BCR-ABL1* cooccur, the case should be diagnosed as *NPM1*-mutated AML. However, the addition of an ABL kinase inhibitor to the treatment should be considered. Similarly, when *NPM1* and *RUNX1* mutations coexist (<1% cases), the patient should classified as *NPM1*-mutated AML.[8] Accordingly, *RUNX1* mutations occurring in *NPM1*-mutated AML are quite different from those classically found in other AMLs, since they are in frame, located outside the RUNT domain, germline, and of unclear functional significance.[14]

Table 1. Main characteristics of *NPM1*-mutated AML

Specific characteristics and comments
Approximately 30-35% of adult AML (50-60% of AML with normal cytogenetic). Less frequent in children (~2-8%).* Female predominance.
BM usually markedly hypercellular. Mostly myelomonocytic (FAB M4) and monocytic (FAB M5), but all FAB categories are represented.
Approximately 23% of cases show multilineage dysplasia.
WBC count may be influenced by *FLT3* mutational status, progressively increasing from *FLT3* wild-type to *FLT3*-ITD^{high}.
Frequent association with extramedullary involvement, especially skin (easily detectable by IHC).
No/low expression of CD34. The rare CD34^{+} leukemic cells carry the *NPM1* mutation. CD34 positivity has been associated with adverse prognosis.
Excellent response to induction chemotherapy.
Relatively good outcome in the absence of *FLT3*-ITD. Prognosis may vary depending upon concomitant mutations.

A synonym of *NPM1*-mutated AML is "AML with cytoplasmic nucleophosmin" (NPM1c^{+}).

FAB, French-American-British.

*Likely because *NPM1* mutations are often preceded by clonal hematopoiesis, which is very rare in children.

At the time we discovered *NPM1* mutations in AML, we also reported for the first time that *NPM1* and *FLT3* internal tandem duplication (ITD) mutations were frequently associated, suggesting that they were mechanistically related.[1] Several investigators have subsequently contributed to demonstrate the biological and clinical importance of this association to the extent that the status of *NPM1* and *FLT3* genes is now among the most important pillars upon which the European LeukemiaNet (ELN) prognostication system is built.[15] The 2017 ELN guidelines[15] (as compared with the 2008 version) recognize 3 (instead of 4) genetic-based risk groups: favorable, intermediate, and high. Another substantial revision has been the introduction of *FLT3*-ITD allelic ratio determined as the ratio of the area under the curve of *FLT3*-ITD and *FLT3* wild-type. *NPM1*-mutated AML without *FLT3*-ITD or with *FLT3*-ITD^{low} (ratio < 0.5) are classified as favorable-risk categories while *NPM1*-mutated AML with FLT3^{high} (ratio > 0.5) is regarded as intermediate risk.[15]

Selection of the best treatment strategy for patients with *NPM1*-mutated AML can be potentially refined combining the ELN prognostication model with the results of MRD evaluated by quantifying *NPM1*mut transcript copies[10] at diagnosis and at selected time points after therapy. In fact, *NPM1* mutations are ideal targets for MRD monitoring, since they are AML specific, frequent, and stable at relapse, and, unlike *DNMT3A, TET2*, or *IDH* mutations, they do not drive clonal hematopoiesis, being a "gatekeeper" for AML.[16-18]

Although therapies specifically directed against the *NPM1* mutant or downstream signaling pathways are not yet available, recent clinical studies have identified drug combinations that may be particularly effective in *NPM1*-mutated AML (eg, venetoclax-based regimens).[19]

Herein, we present 4 illustrative cases of *NPM1*-mutated AML, with the aim to address relevant issues on the diagnosis and therapy of this frequent leukemia.

Scenario 1: Young adult patient with *NPM1*-mutated AML, *FLT3* wild-type, and skin involvement

A 60-year-old man presented with multiple skin nodules without symptoms. A complete blood count (CBC) showed white blood cell (WBC) 1.6×10^9/L, hemoglobin (Hb) 13.8 g/dL, and platelets 167×10^9/L. BM was diffusely infiltrated by myeloid blasts expressing cytoplasmic NPM1 at IHC. *NPM1* mutation A and *FLT3* wild-type were detected; karyotyping was normal. Skin biopsy specimen showed leukemic involvement (Figure 1A-C). A "7+3" induction chemotherapy (CHT) led to complete remission (CR) and disappearance of all skin lesions (Figure 1D-E). RT-qPCR showed 3.9-log reduction of *NPM1*mut transcripts in BM following induction, 4.5-log reduction after the first consolidation, and MRD negativity after the second consolidation cycle. In the following 6 months, the *NPM1*mut transcripts in BM progressively increased (Figure 1F). He received pre-emptive treatment with off-label dactinomycin,[20] achieving a slight MRD decrease (Figure 1F), followed by allogeneic hematopoietic stem cell transplantation (allo-HSCT)[21] from an haploidentical donor. He is now in molecular CR (Figure 1F) >3 years after the allotransplant.

Questions and recommendations The first question is what the optimal frontline therapy for our patient should be. Young adults (≤60 years) with *NPM1*-mutated AML without *FLT3*-ITD should receive induction plus consolidation therapy[15,22-25] (Figure 2). Based on recent studies, the addition of gemtuzumab ozogamicin (GO) to CHT may be of benefit in *NPM1*-mutated AML. A meta-analysis[26] clearly showed a survival benefit for patients with intermediate-risk cytogenetics and *NPM1*-mutated AML due to a reduced relapse risk. This was confirmed in the ALFA0701 trial[27] that also proved the role of GO in serially reducing *NPM1*mut transcripts. The AMLSG study also showed an improved relapse-free survival[28] and a reduced cumulative incidence of relapse in *NPM1*-mutated AML due to deeper reduction of *NPM1*mut transcript levels across all treatment cycles.[29] However, the early primary end point of this study (event-free survival) was not met due to excessive toxicity, possibly related to a combination of the dosing strategy and the inclusion in the regimen of ATRA and etoposide.[28] GO benefit in this study was mostly observed in females ≤70 years without *FLT3*-ITD.[28] Altogether, these findings support the incorporation of GO into the frontline treatment of *NPM1*-mutated AML.

The second question refers to the best postremission therapy in cases like our patient. Allo-HSCT in first CR (CR1) is not recommended for *NPM1*-mutated AML patients without *FLT3*-ITD.[30] Allo-HSCT has been advocated for patients ≤50 years with low predicted transplant-related mortality and an HLA-identical donor.[31] However, allotransplant results in better relapse-free survival, but not overall survival (OS), likely because patients can be salvaged by allo-HSCT in second CR.[31] A suboptimal reduction of *NPM1* transcripts after 2 CHT cycles could potentially help selecting patients with favorable genotype who may benefit from allo-HSCT, but this needs to be prospectively

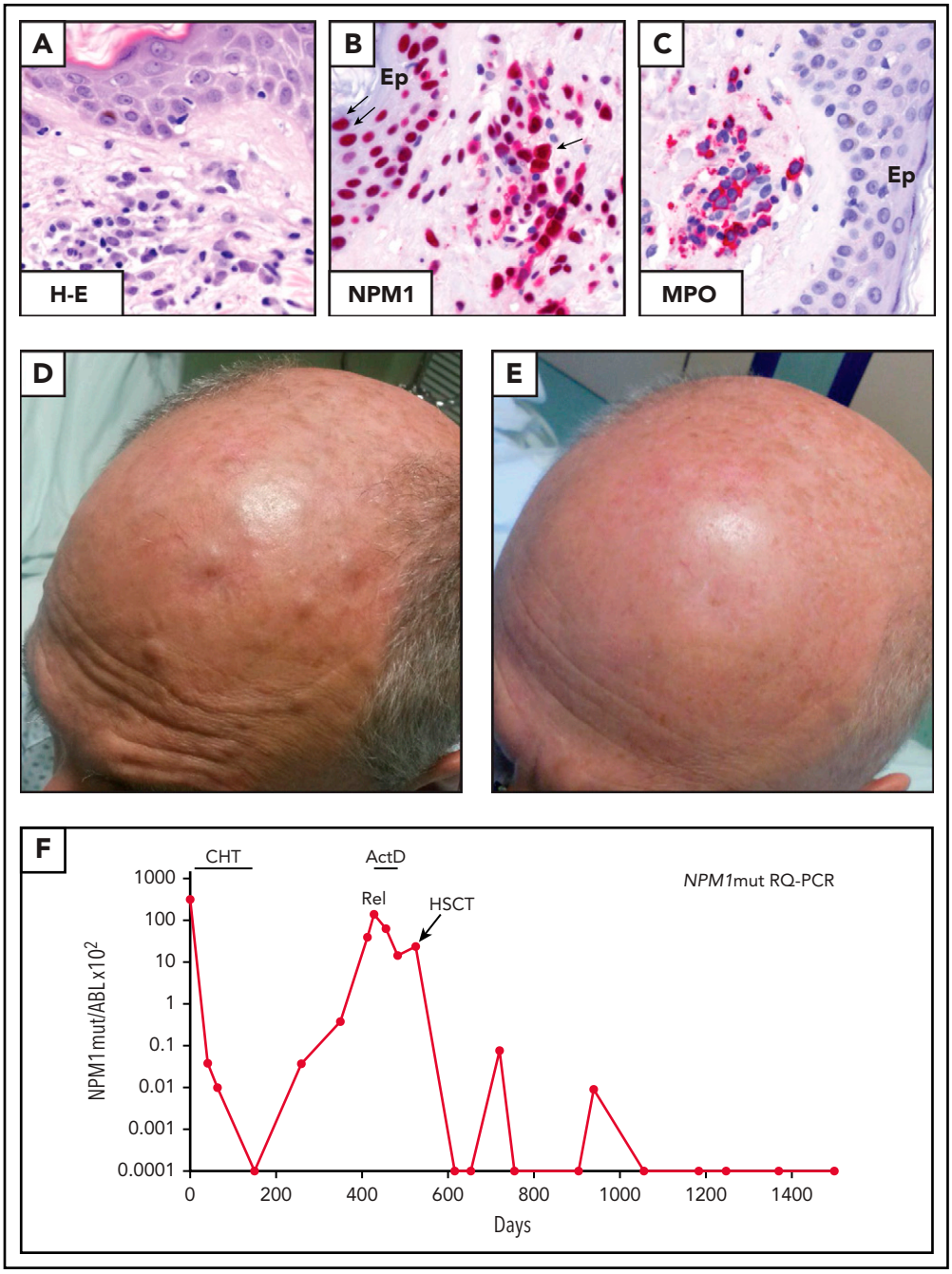

Figure 1. *NPM1*-mutated myeloid sarcoma of the skin. (A) Dermal infiltration by leukemic cells (skin biopsy, B5-fixed; hematoxylin-eosin, original magnification ×400). (B) Leukemic cells exhibit aberrant cytoplasmic expression of NPM1 (arrow). Normal cells of epidermis show nucleus-restricted expression of NPM1 (double arrows) (immune alkaline phosphatase anti-alkaline phosphatase [APAAP] staining, original magnification ×400). (C) Leukemic cells in the derma are myeloperoxidase-positive (APAAP staining, original magnification ×400). Ep, epidermis. (D-E) Cutaneous nodules at diagnosis (D) disappeared after induction chemotherapy (E). (F) Monitoring of *NPM1*mut transcripts during therapy and follow-up (see text). 0.0001 is equivalent to MRD negativity. ActD, dactinomycin; Rel, relapse; RQ-PCR, RT-qPCR.

validated. Under these circumstances, we usually monitor the MRD, and in case of progressive increase of *NPM1*mut transcripts over time, we consider administering CHT in the attempt to reduce MRD before proceeding to allo-HSCT.

A yet-controversial issue remains whether patients with *NPM1*-mutated AML with *FLT3*-ITD^{low}, which also belongs to the ELN favorable-risk category, should undergo allo-HSCT in CR1, as a good outcome of this genotype has been recently questioned in several studies. Straube et al[32] found a lower survival and increased relapse risk compared with other favorable-risk patients. Similarly, Sakaguchi et al[33] reported that *NPM1*-mutated AML patients with *FLT3*-ITD^{low} treated with CHT alone did not show favorable outcome (5-year OS, 41.3%) unless they underwent allo-HSCT in CR1. Conversely, Dohner et al,[25] in a retrospective analysis of the impact of *NPM1*/*FLT3*-ITD genotypes in the RATIFY trial (CHT vs CHT + midostaurin), confirmed the favorable prognosis of *NPM1*-mutated AML with *FLT3*-ITD^{low} treated with CHT + midostaurin (5-year OS, 73%)[25] and found no benefit of allo-HSCT in this setting. Indeed, using the Simon-Makuch plots, the authors

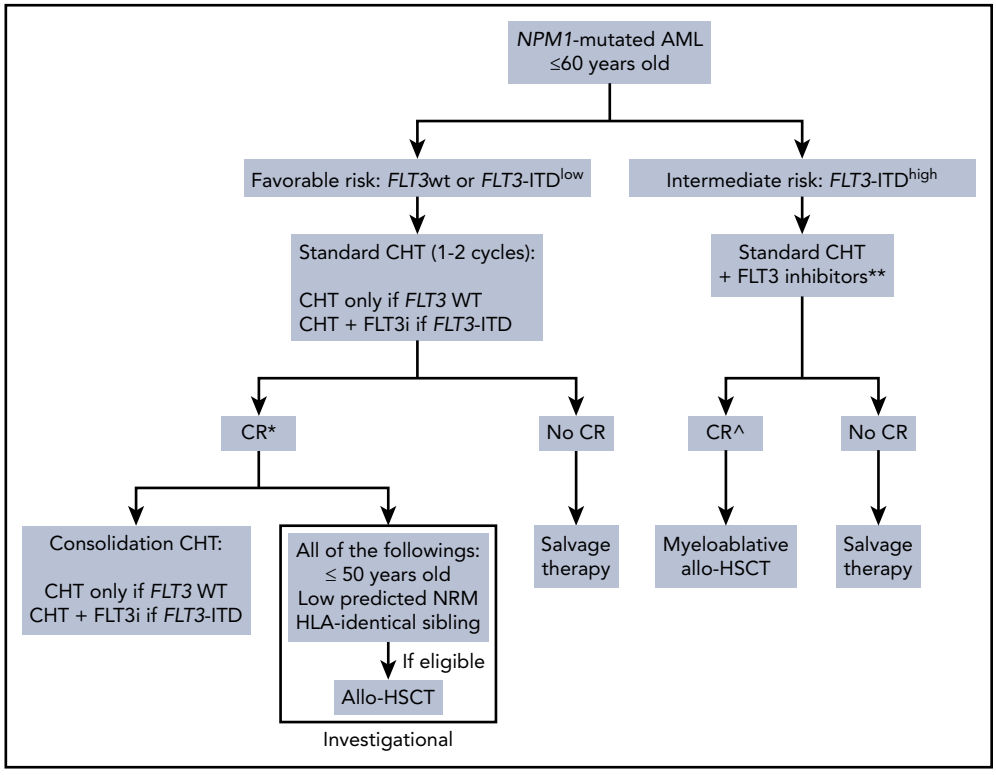

Figure 2. Treatment algorithm for young fit *NPM1*-mutated AML patients with *FLT3*-ITD^{wt}, *FLT3*-ITD^{low}, and *FLT3*-ITD^{high}. All patients with mutated *FLT3* (irrespective of allelic ratio) should receive FLT3 inhibitor plus CHT as induction/consolidation therapy. Some authorities consider allo-HSCT in CR1 for patients with *FLT3*-ITD^{low}, except for cases with ratio <0.1. *Whether patients with suboptimal reduction of *NPM1* transcripts after 2 CHT cycles with or without FLT3 inhibitor (depending on *FLT3* status) may benefit from allo-HSCT should be investigated in prospective clinical trials. **Proceed to allo-HSCT as soon as possible. ^Whether the subset of patients with *FLT3*-ITD^{high} achieving MRD negativity after 2 CHT cycles may avoid allo-HSCT in CR1 should be investigated in prospective clinical trials. FLT3i, FLT3 inhibitor; NRM, nonrelapse mortality; WT, wild-type.

found that allo-HSCT was beneficial only in the ELN adverse risk group. Overall, these findings suggest that the impact of *FLT3*-ITD^{low} in risk stratification may depend on the treatment setting (eg, FLT3 inhibitors). However, some authorities still consider allo-HSCT in CR1, except for patients with a very low *FLT3*-ITD allelic ratio (eg, <0.1). In *FLT3*-ITD^{low} patients treated with standard CHT plus a FLT3 inhibitor, we follow the same policy as in *FLT3* wild-type patients. The decisional algorithm for *NPM1*-mutated AML without *FLT3*-ITD and with FLT3-ITD^{low} is shown in Figure 2.

Based on patient's age and the lack of an HLA-identical donor, we decided not to allotransplant our patient in CR1. Another factor that guided our clinical decision to avoid transplant was the MRD negativity achieved at the end of treatment. Indeed, achieving MRD negativity by RT-qPCR in peripheral blood (PB)[34] or BM[35] or deep reduction in *NPM1*mut transcripts[36-38] (optimal thresholds remain uncertain[39]) at various time points after CHT predicts favorable outcomes. MRD evaluation in PB after 2 CHT cycles appears even more precise than genetic-based prognostication in predicting risk of relapse.[34] Thus, as recommended by ELN,[40] we monitored the level of *NPM1*mut transcripts both in BM and PB every 3 months. This time schedule is based on the observation that kinetics of *NPM1*-mutated AML is quicker than that of *CBF-MYH11* in core-binding factor AML.[41] Progressive MRD increase predicts hematological relapse.[35,40,42] Unfortunately, despite the favorable genotype and the excellent molecular response, our patient showed a progressive increase in *NPM1*mut transcripts yet remained in hematological CR. T lymphocytes reactive against tumor-specific HLA-presented peptides of NPM1 mutants have been demonstrated in *NPM1*-mutated AML patients.[43] Relapse in patients achieving MRD negativity may reflect the inability of the immune system to clear residual leukemic cells (below the threshold of detection by RT-qPCR).

NPM1-mutated AML patients in molecular relapse should be offered an allo-HSCT. Some clinicians wait for hematological relapse while others either proceed directly to allo-HSCT or administer pre-emptive therapy[44,45] with the aim of reducing MRD levels before allo-HSCT, since MRD levels correlate with allotransplant outcomes.[46-48] For this reason, we pre-emptively treated our patient with dactinomycin[20] before allo-HSCT. Other options for pre-emptive therapy in *NPM1*-mutated AML include 5-azacitidine,[49] venetoclax,[50] and immunotherapy.[51] In one study, patients in molecular relapse that received pre-emptive 5-azacitidine showed lower incidence or delayed hematological relapse compared with historical controls.[49] In another study, *NPM1*-mutated AML patients without *FLT3*-ITD or with *FLT3*-ITD^{low} did better if treated at molecular failure rather than at hematological relapse (80% vs 40%, 2-year OS, respectively).[45] However, more studies are required to establish the best therapeutic strategy in the setting of molecular relapse.

Another important point highlighted in this case refers to the diagnosis and clinical significance of skin involvement in *NPM1*-mutated AML. Immunohistochemical detection of cytoplasmic NPM1 helps establishing leukemic skin involvement, a relatively common event in *NPM1*-mutated AML.[52] Based on a large study showing no prognostic significance of extramedullary disease in AML (although mutations were not analyzed),[53] we do not regard skin involvement at presentation as a poor prognostic factor in *NPM1*-mutated AML. Thus, clinical decisions in our patient were exclusively based on ELN risk stratification and MRD response.

Scenario 2: Young adult patient with *NPM1*-mutated AML and high *FLT3*-ITD allelic ratio (*FLT3*-ITD^{high})

A 58-year-old man presented with fatigue, gingival hyperplasia. and bleeding. CBC showed WBC 52.6 × 10^9/L, Hb 9.4 g/dL, and

platelets 48 × 10^9/L. BM examination was consistent with *NPM1*-mutated AML, as proven by expression of cytoplasmic NPM1. Diagnosis was confirmed by the identification of *NPM1* mutation A and *FLT3*-ITD (ratio 0.9). The BM karyotype was normal. The patient received a 7+3 induction regimen, achieving CR and only a 1.7-log reduction of *NPM1*mut transcripts, with *FLT3*-ITD still detectable with a ratio of 0.01. An additional 2.5-log reduction of *NPM1*mut copies and *FLT3*-ITD negativity was achieved after 2 consolidation cycles. The patient underwent a haploidentical HSCT. He is now in molecular CR, 5 years after the allotransplant.

Questions and recommendations Currently, *NPM1*-mutated AML patients with *FLT3*-ITD^{high} (ratio ≥ 0.5) should receive conventional chemotherapy plus a FLT3 inhibitor[25,54] (not approved at the time our patient was treated) (Figure 2). As discussed in scenario 1, these patients may also benefit from GO incorporation into frontline therapy.[27]

Allo-HSCT in CR1 represents the best therapeutic option in *NPM1*-mutated AML patients with *FLT3*-ITD^{high}, like our case[15,31,38,55-58] (Figure 2), although some authors claim it may be questionable in patients achieving postremission *NPM1*mut MRD negativity.[59] Our policy is to go to allo-HSCT as soon as possible. If >3-log reduction of *NPM1*mut in BM is achieved after 1 or 2 CHT cycles, we usually proceed to myeloablative allo-HSCT, possibly avoiding repeated rounds of consolidation CHT. However, we decided to administer 2 consolidation cycles to our patient, because he achieved suboptimal reduction of molecular MRD after induction. This decision was based on studies showing that *NPM1*-mutated AML patients MRD-positive immediately before allo-HSCT usually have poor outcome,[46-48] although they still do better with allotransplant than with CHT alone,[38,58] especially those with >4-log MRD reduction in PB.[38] Furthermore, in a recent study,[48] *NPM1*-mutated *FLT3*-ITD-positive patients with any level of pretransplant MRD positivity showed a 2-year OS of ~20%. Conversely, patients with pretransplant MRD negativity had a 2-year OS survival approaching 80%, irrespective of the *FLT3*-ITD status and intensity of transplant conditioning regimen.[48] Similar good outcome was also observed in *NPM1*-mutated *FLT3*-ITD-negative patients with low levels of pretransplant MRD (ie, <200 copies/10^5 *ABL* in PB or <1000 copies/10^5 *ABL* in BM).[48] Although reducing pretransplant MRD as much as possible (ideally achieving negativity) in *NPM1*-mutated *FLT3*-ITD^{high} seems reasonable, the feasibility and clinical significance of this approach remains unknown.

Scenario 3: Old unfit patient with *NPM1*-mutated AML without *FLT3*-ITD plus multilineage dysplasia

A 76-year-old man presented with pneumonia and fever. CBC revealed WBC 0.87 × 10^9/L, Hb 8.0 g/dL, and platelets 121 × 10^9/L. The BM was infiltrated by AML (blasts 40%) with dysplasia in >50% of myeloid and erythroid progenitors. Megakaryocytes were also dysplastic. Cells of all 3 hematopoietic lineages were cytoplasmic positive for NPM1 (Figure 3A-D). Molecular studies identified a *NPM1* mutation, type A and *FLT3* wild-type. The BM karyotype was normal. As the patient was unfit for intensive CHT, he was treated with dactinomycin within a clinical trial (EudraCT 2014-003490-41), achieving CR and >3-log reduction in *NPM1* transcripts. Ten months later, the patient relapsed. He received off-label venetoclax plus the hypomethylating agent (HMA) 5-azacytidine, achieving CR and marked reduction in *NPM1*mut transcripts. He is now at his fifth cycle of venetoclax plus HMA.

Questions and recommendations The low WBC count and morphological features observed in our patient poses the problem of differential diagnosis between *NPM1*-mutated AML with multilineage dysplasia and AML with myelodisplasia-related changes (AML-MRC). Distinction of these 2 entities is critical, since postremission therapy significantly differs between favorable-risk *NPM1*-mutated AML with multilineage dysplasia and AML-MRC.[60]

In our experience, low WBC count is still consistent with a diagnosis of *NPM1*-mutated AML, especially in cases with wild-type *FLT3*. According to the 2017 WHO classification, multilineage dysplasia is defined as the presence of dysplasia in at least 2 BM cell lines. This occurs in ~23% of patients with *NPM1*-mutated AML.[61] In BM biopsies of *NPM1*-mutated AML patients, megakaryocytes are often increased in number and dysplastic,[62] but, unlike AML-MRC or myelodysplastic syndrome (MDS), they are consistently CD34 negative. This is in keeping with our finding that *NPM1* mutant A perturbs megakaryopoiesis in a conditional mouse model[63] and may also explain the relatively high platelets count in our patient.

The demonstration that *NPM1*-mutated AML with and without multilineage dysplasia show the same immunophenotype, gene expression profile, and clinical outcome[61] led to a major change in the 2017 edition of WHO classification (as compared with the previous 2010 version). Specifically, when *NPM1* mutation and multilineage dysplasia coexist, the genetic lesion supersedes morphology and the case is diagnosed as *NPM1*-mutated AML.[8,61] Conversely, a previous history of MDS or the finding of MRC-related cytogenetic abnormalities, even in the presence of *NPM1* mutation, are diagnostic of AML-MRC. Our patient was diagnosed as *NPM1*-mutated AML, since multilineage dysplasia but no history of MDS or specific cytogenetic abnormalities were found. A diagnostic algorithm for distinguishing *NPM1*-mutated AML with multilineage dysplasia from AML with MRC is shown in Figure 3E.

What is the best therapy available for this patient? Older unfit *NPM1*-mutated AML patients rarely show durable response to single HMA.[64] Thus, combining venetoclax with a HMA (not yet approved by the Italian Drug Agency when we first treated the patient) is now the standard of care, inducing CR in 70% to 90% of *NPM1*-mutated AML patients[65,66] (Figure 4). Most patients achieve CR after 1 cycle. Prophylactic measures should be taken to prevent the venetoclax-induced tumor lysis syndrome, although the risk is lower than in chronic lymphocytic leukemia.[67]

Main side effects of venetoclax plus HMAs are hematological, with ~40% of cases showing cytopenias and ~30% grade ≥3 infectious complications.[68] These may require dose reductions or interruption of venetoclax. Other common nonhematological adverse events of venetoclax plus 5-azacytidine are gastrointestinal.[66] However, mortality at 30 days was only 7% with this combo,[66] clearly indicating the safety of this regimen in unfit patients. Venetoclax is a cytochrome P450 3A4 substrate. Thus, its dose should be reduced by at ≥75% when used in combination with posaconazole, a strong cytochrome P450 3A4 inhibitor commonly used for antifungal prophylaxis in AML.[67]

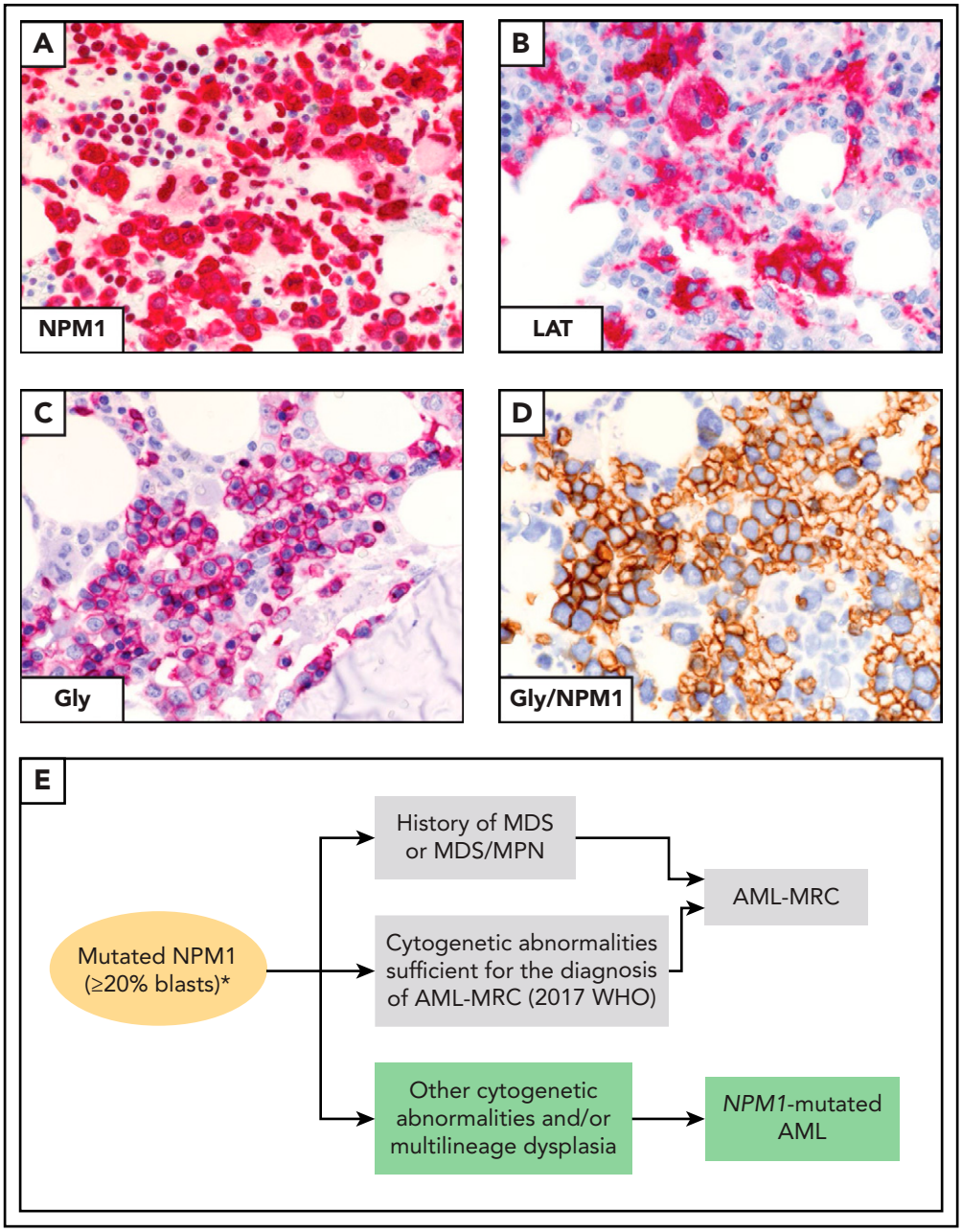

Figure 3. Multilineage dysplasia in *NPM1*-mutated AML. (A) Aberrant cytoplasmic expression of NPM1 in leukemic cells of the 3 hematopoietic cell lineages. (BM biopsy, APAAP staining; original magnification ×400). (B) An area showing several dysplastic megakaryocytes detected by immunostaining against LAT (linker for activation of T cells) (BM biopsy, APAAP staining; original magnification ×400). (C) An area occupied by numerous leukemic cells of erythroid cell lineage that show surface expression of glycophorin (Gly; BM biopsy, APAAP staining; original magnification × 400). (D) Leukemic cells stained for glycophorin (brown) and cytoplasmic NPM1 (blue) (BM biopsy, immunoperoxidase [brown]/APAAP [blue]; original magnification ×400). (E) Diagnostic algorithm for the differential diagnosis between *NPM1*-mutated AML and AML-MRC. *No prior cytotoxic or radiation therapy; no recurrent cytogenetic abnormalities defining distinct AML entities of 2017 WHO classification.

Venetoclax-based regimens may be also considered as a temporary alternative to intensive CHT in older fit *NPM1*-mutated AML patients during the COVID-19 pandemic.[69,70] Under these circumstances, monitoring of MRD is highly recommended, although the optimal time points for assessment of *NPM1*mut transcripts level needs yet to be established for venetoclax-based regimens.[71]

Older fit patients (>60 years) with *NPM1*-mutated AML should be treated intensively, since they respond better than other genotypes to induction-consolidation CHT[72] (Figure 4). However, the outcome remains adverse (15% to 20% OS) independently of the *FLT3* status.[72,73] In fact, patients >65 years with *NPM1*-mutated AML without *FLT3*-ITD exhibit a prognosis poorer than younger patients with the same genotype (2-year OS, 27% vs 70%, respectively).[74] Older *NPM1*-mutated AML patients with *FLT3*-ITD^{low} also experience poor outcomes.[32] However, this scenario may change rapidly. Improvement in event-free survival has been reported in older AML patients with *FLT3*-ITD following the addition of midostaurin to CHT.[75] Moreover, venetoclax-based regimens have the potential to challenge standard CHT as frontline treatment in *NPM1*-mutated AML.[65] Recently, venetoclax in combination

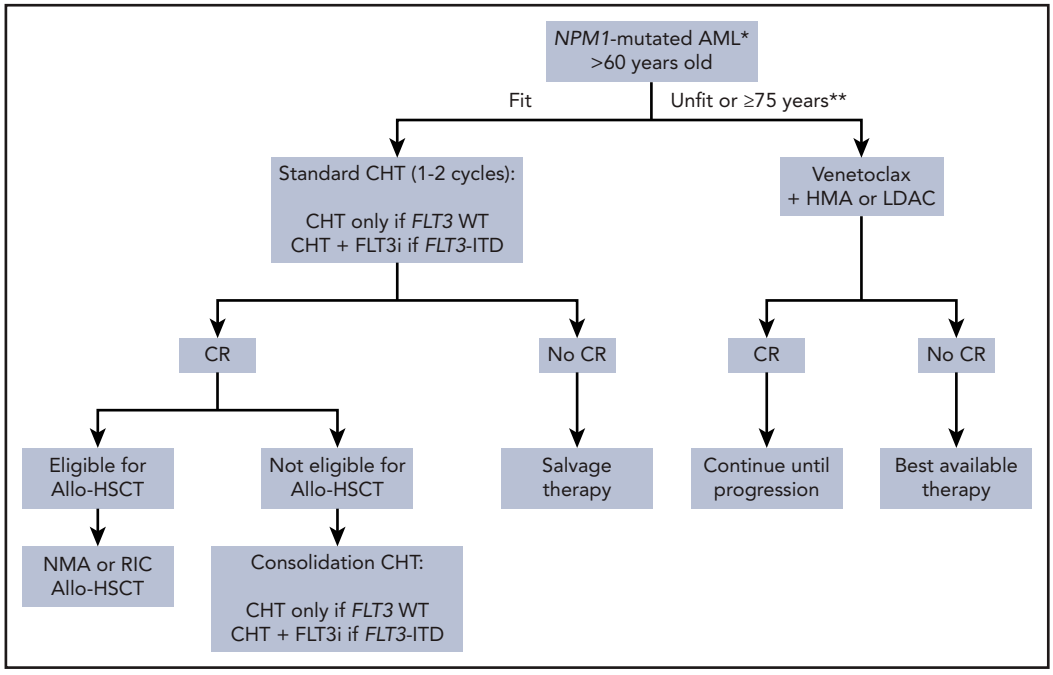

Figure 4. Treatment algorithm for older patients with *NPM1*-mutated AML. *Include *NPM1*-mutated AML independently by the *FLT3*-ITD that does not appear prognostically relevant in older patients.[32,74] **Venetoclax-based regimens are approved for fit AML patients ≥75 years by the Italian Drug Agency. During the COVID-19 pandemic, venetoclax-based regimens may be used temporarily in alternative to intensive CHT in older fit *NPM1*-mutated AML patients,[69] to help bridge patients through the pandemic with the aim of subsequently delivering definitive therapy, including allo-HSCT, if required. LDAC, low-dose cytarabine; NMA, nonmyeloablative; RIC, reduced intensity conditioning.

with "5+2" induction CHT (CAVEAT trial) followed by 4 consolidation cycles was found to be safe in fit older patients with AML.[76] Deep molecular responses were achieved in most cases of *NPM1*-mutated AML. The major limiting toxicity in the trial was prolonged thrombocytopenia. This is probably due to the fact that venetoclax may inhibit *p*-glycoprotein, enhancing intracellular accumulation of idarubicin with consequent toxicity on normal hemopoietic stem cells. *NPM1*-mutated AML without *FLT3*-ITD can benefit of reduced intensity conditioning[77] or nonmyeloablative[78] allo-HSCT (Figure 4).

Scenario 4: *NPM1*-mutated AML relapsing with therapy-related myelodysplasia lacking *NPM1* mutation

A 68-year-old women presented with fatigue, dyspnea and chest pain. CBC showed WBC 3.9 × 10^9/L, Hb 9.7 g/dL, and platelets 178 × 10^9/L. The BM was massively infiltrated by blasts with myelomonocytic appearance showing cytoplasmic NPM1 and coexpressing myeloperoxidase and macrophage-restricted CD68, while CD34 was negative. *NPM1* mutation A and *FLT3* wild-type were detected. BM karyotype was normal. A 7+3 induction regimen resulted into CR with a 4-log reduction of *NPM1*mut transcripts in BM (Figure 5A). She received 2 cytarabine consolidation cycles, achieving MRD negativity but, after 14 months, developed fever and thrombocytopenia (54 × 10^9/L). BM examination showed MDS with nuclear expression of NPM1. *NPM1*mut transcripts were undetectable (Figure 5A), while karyotyping and fluorescence-activated cell sorting revealed del(5q), −7 and monoallelic deletion of *TP53*. She received 2 cycles of 5-azacytidine (Figure 5A) with platelet recovery; del(5q) and −7 disappeared, while *TP53* deletion persisted in 90% of nuclei. Because of high-risk cytogenetics,[8] she underwent an haploidentical HSCT (Figure 5A) and is now in cytogenetic CR 2.5 years after the allotransplant.

Questions and recommendations This case addresses the issue of clonal evolution in *NPM1*-mutated AML. Approximately 10% of *NPM1*-mutated AML patients relapse with no detectable *NPM1* mutation.[79] These cases represent second AML emerging after the eradication of the original *NPM1*-mutated clone, as proven by different mutational patterns at diagnosis and relapse.[80,81] This event may be facilitated by preexisting clonal hematopoiesis and usually occurs after several years.[79-81] The second AML may represent either a de novo or therapy-related event (Figure 5B). In our patient, the morphology and fluorescence-activated cell sorting at the time of relapse were diagnostic of therapy-related MDS (t-MDS).[8]

Except for the short time (~1 year) before t-MDS development, our case resembles those reported by Herold et al.[82] Specifically, 5 patients (median age, 58 years; age range, 47-74 years) initially diagnosed as *NPM1*-mutated AML developed t-MDS after a median of 46 months (range, 28-92 months) remaining MRD negative for *NPM1*mut. In 3/5 cases, the karyotype was normal while 2 cases showed, like our patient, chromosome 7 abnormalities.[82] Mutational analysis exhibited persistence of clonal hematopoiesis that may have favored the emergence of t-MDS,[82] as also reported in another study.[83] We had no information on preleukemic clonal hematopoiesis in our patient.

Future perspectives and conclusions

Great progress has been made during the past 15 years in the diagnosis and therapy of *NPM1*-mutated AML, but there are a

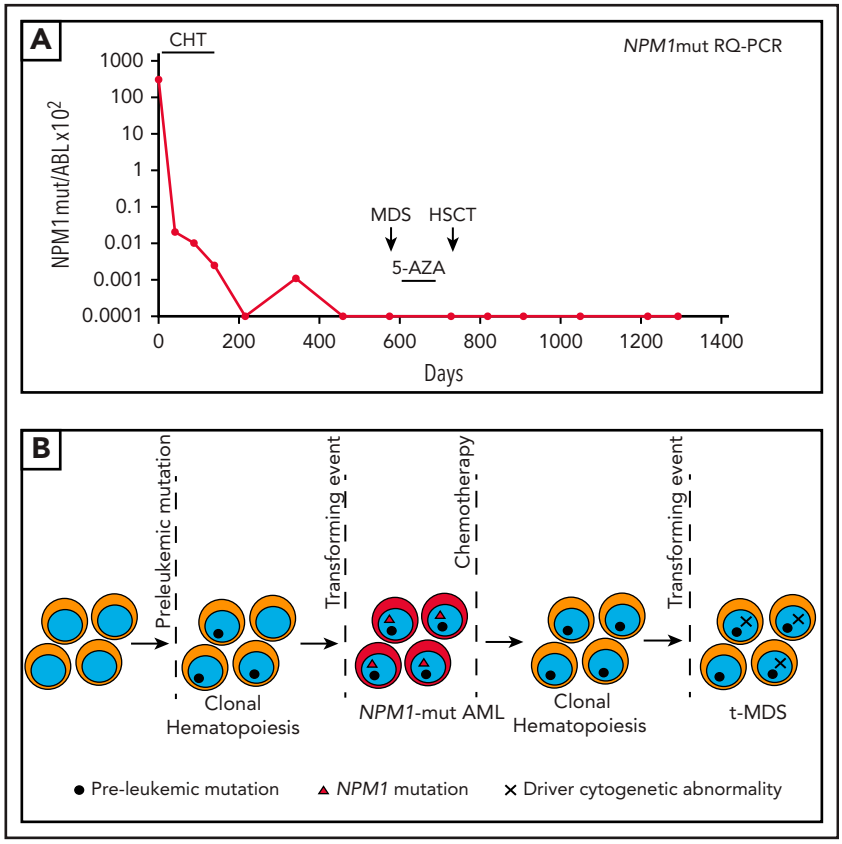

Figure 5. *NPM1*-mutated AML relapsing as therapy-related MDS. (A) Monitoring of *NPM1*mut transcripts during therapy and follow-up (see text). 0.0001 is equivalent to MRD negativity. (B) Cartoon depicting the clonal architecture of the BM over time in the patient described in scenario 4. Absence of *NPM1* mutations and presence of MRC-related cytogenetic abnormalities at relapse demonstrated the development of a second myeloid neoplasm (t-MDS). 5-AZA, 5-azacitidine; RQ-PCR, RT-qPCR.

number of issues that remain to be clarified and objectives that need to be accomplished.

According to the 2017 WHO classification,[8] the percentage of blasts required for diagnosing *NPM1*-mutated AML must be ≥20%.[8] This criterion differs from that adopted for AML with t(8;21), inv(16), t(16;16), and t(15;17), which can be diagnosed regardless of blast cell count. This is due to the report of very rare cases of *NPM1*-mutated MDS and chronic myelomonocytic leukemia (CMML).[84,85] However, *NPM1* mutations in CMML associate with normal cytogenetics, "dysplastic phenotype," *DNMT3A* mutations,[84] higher risk of blastic transformation, and outcomes poorer than CMML without *NPM1* mutations.[84] Moreover, dramatic monocytosis is sometimes observed in patients with *NPM1*-mutated AML (especially M5b), in keeping with findings in immunocompromised mice transplanted with $CD34^+$ HSCs from *NPM1*-mutated AML patients.[86] Similarly, cases of *NPM1*-mutated MDS show features typical of AML, including good response to intensive chemotherapy.[85,87] Thus, *NPM1* mutations may define AML, irrespective of blast percentage.[88] Under these circumstances, we recommend performing a BM biopsy and IHC for cytoplasmic NPM1, since these procedures together allow to evaluate the percentage of blasts better than conventional morphology and immunophenotype. We currently treat patients with *NPM1* mutations and <20% of blasts as *NPM1*-mutated AML, although we recognize that this still represents a controversial issue that requires further studies.

The ELN model is an oversimplification of the real world, since the prognosis of *NPM1*-mutated AML also depends on comutated genes other than *FLT3*.[89] For example, the *NPM1/N-RAS*, *NPM1/RAD21*[89] or *NPM1/FLT3*-tyrosine kinase domain[90] genotypes seem to have a relatively favorable outcome, while the *NPM1/DNMT3A/FLT3*-ITD[89] and *NPM1/WT1*[91] genotypes show a particularly poor prognosis. Other genotypes occur at very low frequency, making the study of their prognostic impact difficult. It is likely that future versions of 2017 ELN model will be updated according to therapeutic progresses, such as taking into consideration the results achieved with FLT3 inhibitors that were not available when the current ELN risk categorization was established.

MRD evaluation also plays an important role in proper relapse-risk estimation in *NPM1*-mutated AML. Today, RT-qPCR[10] represents the gold standard for assessing levels of *NPM1*mut transcripts, since it is inexpensive and can be used for MRD monitoring in >90% of *NPM1*-mutated AML cases. However, sensitivity of next-generation sequencing for MRD monitoring has improved,[92-95] and it is expected that this technique will be increasingly used in the future, especially for rare *NPM1* mutations. Currently, ELN[15] recommends using next-generation sequencing only in clinical trials. Standardizing of *NPM1* MRD measurement and better defining its impact at different time points after therapy as well designing *NPM1* MRD-based prospective clinical trials is also warranted.

Venetoclax, in combination with CHT, should be further investigated in *NPM1*-mutated AML. Also, unfit *NPM1*-mutated AML patients relapsing after venetoclax-based regimens represent a medical need, and further studies should be directed to better understand the mechanisms of resistance to venetoclax. *FLT3* mutations (frequent in *NPM1*-mutated AML) may promote resistance to venetoclax by enhancing expression of other members of the

BCL-2 family, including BCL-XL and MCL-1.[96] Therefore, there is a rationale in using venetoclax plus FLT3 inhibitors in *NPM1*-mutated AML comutated for *FLT3*. Phase 1/2 studies combining venetoclax with gilteritinib (NCT03625505) or quizartinib (NCT03735875) are ongoing.

Promising future new agents in *NPM1*-mutated AML include XPO1[5] and MLL-Menin[97,98] inhibitors, alone or in combination with FLT3 inhibitors[99] or venetoclax[100] and drugs targeting the interaction between NPM1 and its ligands.[101]

In conclusion, a better knowledge of the mechanisms underlying leukemic transformation mediated by mutant NPM1 and the design of MRD-focused clinical trials are expected to further improve and personalize the therapy of *NPM1*-mutated AML patients.

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Authorship

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Footnote

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