RESEARCH ARTICLE

Nucleophosmin1 and isocitrate dehydrogenase 1 and 2 as measurable residual disease markers in acute myeloid leukemia

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Abstract

Monitoring measurable residual disease (MRD) in acute myeloid leukemia (AML) plays an important role in predicting relapse and outcome. The applicability of the leukemia-initiating nucleophosmin1 (NPM1) gene mutations in MRD detection is well-established, while that of isocitrate dehydrogenase1/2 (IDH1/2) mutations are matter of debate. The aim of this study was to investigate the stability of NPM1 and IDH1/2 mutations at diagnosis and relapse retrospectively in 916 adult AML patients. The prognostic value of MRD was evaluated by droplet digital PCR on the DNA level in a selected subgroup of patients in remission. NPM1 re-emerged at relapse in 91% (72/79), while IDH1/2 in 87% (20/23) of mutation-positive cases at diagnosis. NPM1 mutation did not develop at relapse, on the contrary novel IDH1/2 mutations occurred in 3% (3/93) of previously mutation-negative cases. NPM1 MRD-positivity after induction (n = 116) proved to be an independent, adverse risk factor (MRD pos 24-month OS: 39.3 ± 6.2% versus MRD neg: 58.5 ± 7.5%, p = 0.029; HR: 2.16; 95%CI: 1.25–3.74, p = 0.006). In the favorable subgroup of mutated NPM1 without fms-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) or with low allelic ratio, NPM1 MRD provides a valuable prognostic biomarker (NPM1 MRD pos versus MRD neg 24-month OS: 42.9 ± 6.7% versus 66.7 ± 8.6%; p = 0.01). IDH1/2 MRD-positivity after induction (n = 62) was also associated with poor survival (MRD pos 24-month OS: 41.3±9.2% versus MRD neg: 62.5±9.0%, p = 0.003; HR 2.81 95%CI 1.09–7.23, p = 0.032). While NPM1 variant allele frequency decreased below 2.5% in remission in all patients, IDH1/2 mutations (typically IDH2 R140Q) persisted in 24% of cases. Our results support that NPM1 MRD even at DNA level is a reliable prognostic factor, while IDH1/2 mutations may represent pre-leukemic, founder or sub- clonal drivers.
**Introduction**

Acute myeloid leukemia (AML) is an aggressive hematological malignancy with a rapidly evolving treatment paradigm. Although the majority of patients remain incurable, long-term remissions can be achieved in roughly one-third of these patients. The identification of prognostic markers bears outstanding relevance for optimizing treatment strategy. Measurable residual disease (MRD) after induction therapy and before hematopoietic stem cell transplantation is an independent, post-diagnosis prognostic indicator of relapse and survival. The application of molecular genetics and multiparametric flow cytometry are recommended for monitoring. Requirements for a reliable molecular genetic MRD marker are the following: (i) mutation burden fluctuates in parallel with leukemic tumor burden: present at disease onset, disappearing in remission and re-emerging at relapse, (ii) available method with the capability of achieving high sensitivity [1–3].

Nucleophosmin1 (NPM1) mutations are among the most frequently detected genetic alterations in AML (present in 25–35% of primary AML) defining a separate disease entity. NPM1 frameshift mutations result in altered protein termination, loss of nuclear localization signals, and consequential abnormal cytoplasmic localization of the mutant protein [4–6]. Isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) mutations occur in 7–14% and 8–19% of AML cases respectively. The gain-of-function mutations result in the production of an oncometabolite with consequential hypermethylation, gene expression alterations and impaired hematopoietic differentiation [4, 7].

NPM1 alterations were reported as definite leukemia-founder mutations and optimal MRD markers. On the other hand contradictory data exist, whether IDH1 and IDH2 mutations represent pre-leukemic, or dominant clone mutations, therefore their value in MRD monitoring is not well established [3, 8]. In our study, we aimed to correlate NPM1 and IDH1 and IDH2 mutational variant allele frequencies at diagnosis, remission and relapse to investigate the potential application of these mutations in MRD monitoring.

**Material and methods**

**Patients**

The study included 916 adult patients (449 males/467 females, median age at diagnosis 54 years; range: 16–94), consecutively diagnosed with AML between January 2001 and June 2020 in our Institute (Department of Hematology and Stem Cell Transplantation, Central Hospital of Southern Pest National Institute of Hematology and Infectious Diseases, Budapest, Hungary). In this patient cohort 253 patients were NPM1, 68 IDH1 and 94 IDH2 mutations positive (74 patients carried both NPM1 and IDH1/2 mutations). A significant proportion of patients 81% (n = 746/916) received curative treatment, out of which 26% (n = 176/746) was treated by allogeneic hematopoietic stem cell transplantation (HSCT). MRD monitoring was retrospectively evaluated in a selected subgroup of 116 NPM1 (51 male/65 female, median age at diagnosis 48 years), and 62 IDH1/2 positive patients (23 male/39 female, median age was 49 years).

The inclusion criteria for the MRD monitored subgroup were the following: (i) curative chemotherapy; (ii) morphologic leukemia-free state (MLFS) after induction [1]; (iii) available DNA sample at diagnosis, after induction, and/or before HSCT. Patients with palliative therapy, death in aplasia or death from indeterminate cause, no MLFS after 2 courses of intensive induction treatment; unavailable DNA sample, or patients with rare undetectable NPM1 or IDH1/2 mutation were excluded from MRD evaluation. MRD was determined after induction and one month before HSCT if DNA samples were available. Data from AML patients diagnosed between 2001 and 2009 have already been reported in an earlier study [9] and IDH1/2...
data between 2001–2018 were presented in a Hungarian report [10]. Data collection was performed retrospectively. Definitions of fms-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) low and high allelic ratio, MLFS, overall survival (OS) and relapse-free survival (RFS) were described by European LeukemiaNet (ELN) 2017 recommendations [1]. The study was in accordance of the Declaration of Helsinki and was approved by the Institutional Review Board of Central Hospital of Southern Pest National Institute of Hematology and Infectious Diseases. Written informed consent was provided by all patients.

**Molecular genetic methods**

Genomic DNA and RNA were isolated from bone marrow samples drawn at diagnosis, remission and relapse. Screening for hotspot mutations were performed from genomic DNA, at the time points of diagnosis and repeatedly at relapse by fragment analysis in case of NPM1 (NPM1 diagnosis n = 916, relapse n = 161 if DNA was available); [11], and by high-resolution melting (HRM) or allele specific PCR in case of IDH1/2 (diagnosis n = 842, relapse n = 116 if DNA was available) [9]. Positive cases were monitored with droplet digital PCR (ddPCR) after induction therapy (NPM1 n = 116; IDH1/2 n = 62; double positive = 33), before HSCT (1–30 days before; NPM1 n = 38; IDH1/2 n = 22), if DNA was available at that time point. Mutant NPM1 RNA expression was also tested at diagnosis and after induction therapy (n = 39).

Diagnosis and follow-up samples of NPM1 as well as IDH1 and IDH2 positive AML patients were investigated by ddPCR. For NPM1 mutation detection primer and probe sequences are summarized in S1 Table [12–14]. NPM1 type-A (c.860_863dupTCTG, p.Trp288CysfsTer12) specific reverse primer was described by Gorello *et al.* [13, 15]. A degenerate R primer (type-N) reported by Mencia-Trinchant *et al.* [14] was applied to detect NPM1 mutations at the same position with different nucleotide insertions (c.860_863dupNNNN, p.Trp288CysfsTer12, referred as NPM1 type-N mutation in this study). GAPDH was used as the reference gene for the assay for DNA [16], and ABL1 for RNA [17]. Reactions were performed using Supermix for Probes (no dUTP) (BioRad), 900 nM of each primer, 250 nM of each probe, 100 ng DNA or 240 ng cDNA per well. For genomic DNA, assays were designed by Bio-Rad for the detection of the most common IDH1/2 mutations (IDH1 R132C ID: dHsaMDV2010053, R132H ID: dHsaMDV2010055 and IDH2 R140Q ID: dHsaMDV2010057, R172K ID: dHsaMDV2010059). The PCR program started with an initial denaturation at 98˚C for 10 min, 40 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C (for DNA) and at 60˚C (for RNA) for 60 sec followed by enzyme deactivation at 98˚C for 10 min. The QX200 Droplet Digital PCR System and QuantaSoft Software (Version 1.7.4.0917, BioRad) were used for the evaluation of the results.

The ddPCR measurements were acceptable if: (i) reference copies or total copies > 32,000, (ii) total droplet count >15,000; (iii) empty droplets > 100. MRD samples were measured in duplicate wells to achieve optimally more than 4.5-log sensitivity (at least 32,000 copies of reference gene) [18]. The ddPCR measurements were also performed in 20–35 mutation negative controls to determine the limit of blank (LoB = mean negative samples + 1.645x standard deviation [SD]) and to determine the limit of detection (LoD = mean negative samples + 3.3xSD) [19, 20]. Variant allele fraction (VAF) lower, than 2.5% correspond to <5% (pre)leukemic cells. Samples taken during MLFS (bone marrow blasts <5%; absence of blasts with Auer rods; absence of extramedullary disease) displaying >2.5% VAF were categorized as persisting preleukemic clones.

**Statistical analysis**

Categorical variables were compared by the Fisher’s exact test, continuous variables by Mann-Whitney tests. Kaplan-Meier method with log-rank statistics were used to calculate OS and
RFS [1]. After induction OS were calculated from the time point of diagnosis, RFS from remission irrespective from performing HSCT. Regarding pre-transplant MRD monitoring, comparisons of OS and RFS were performed from the time point of HSCT. Following univariate analysis, age, cytogenetics, \(\text{FLT3-ITD}\) allelic ratio [1], \(\text{NPM1}\), white blood cell count (WBC) at diagnosis, and MRD status were included in a Cox proportional hazard model for OS and RFS. Hazard ratios (HR) and 95% confidence interval (95%CI) values were calculated. In order to identify the cut off discriminating between low and high MRD burden groups, HRs for OS were compared at six different limits (0.05%; 0.1%; 0.2%; 0.5%; 1% and 2%) for \(\text{NPM1}\) type-A and type-N separately and combined [21]. P values below 0.05 were considered as statistically significant. For the statistical analysis SPSS Statistics version 22 (Armonk, NY) was applied.

**Results**

**Occurrence of \(\text{NPM1}\), \(\text{IDH1/2}\) mutations in the total AML cohort**

This study included 916 adult AML patients (S2 Table). Cytogenetic results were available for 94% (\(n=861\)) of patients: favorable (\(n=136\); 16%), intermediate (\(n=507\); 59%) and adverse (\(n=218\); 25%) ELN 2017 cytogenetic risk categories were identified. \(\text{NPM1}\) mutation occurred in 28% (\(n=253/916\)), \(\text{FLT3-ITD}\) in 25% (\(n=226/916\)); \(\text{FLT3}\) tyrosine kinase domain mutations (\(\text{FLT3-TKD}\)) in 8% (\(n=71/910\)); \(\text{IDH1}\) in 8% (\(n=68/842\)) and \(\text{IDH2}\) in 11% (94/842). \(\text{IDH1}\) R132H associated with \(\text{NPM1}\) positivity more commonly than other \(\text{IDH1}\) R132 codon mutations: 90% (\(n=27/30\)) versus 26% (\(n=10/38\)), \(p<0.0001\). Also \(\text{IDH2}\) R140Q co-occurred with \(\text{NPM1}\) in 49% (\(n=37/76\)), while R172K never associated (\(p<0.0001\)).

In the \(\text{NPM1}\)-positive cohort, 211 patients were treated with curative intent, out of which remission (MLFS) was reached in 174 cases (Fig 1). The stability of \(\text{NPM1}\) mutation during disease evolution was studied with 79 paired \(\text{NPM1}\) mutant samples drawn at diagnosis and relapse. The \(\text{NPM1}\) mutation re-emerged at relapse in 91% of \(\text{NPM1}\) positive cases (\(n=72/79\)). Time period from diagnosis till relapse was not significantly longer in cases where \(\text{NPM1}\) was undetectable at relapse compared to cases with persistent \(\text{NPM1}\) mutation at relapse [median 7.1 month (range: 0.1–172.2 month) versus 6.6 month (range: 2.2–152.9 month) respectively, \(p=0.46\)]. All seven patients with clonal \(\text{NPM1}\) regression had normal karyotype at the time of diagnosis; one patient out of five with karyotyping available at relapse had clonal evolution (trisomy 8). None of our \(\text{NPM1}\) negative AML cases gained \(\text{NPM1}\) mutation positivity at relapse.

![Fig 1. Clinical characteristics of \(\text{NPM1}\) positive AML patients. *Remission was defined as morphologic leukemia-free state (MLFS) after induction. †DNA not available at the time point of relapse.](https://doi.org/10.1371/journal.pone.0253386.g001)
among the 82 NPM1 negative patients, where samples at diagnosis and relapse were available at both time points.

In the IDH1/2-positive cohort (n = 162), 132 patients were treated with curative intent, out of which remission (MLFS) was reached in 90 cases (Fig 2). IDH1/2 mutations were undetectable at relapse in 13% of the IDH1/2-positive cohort with available DNA (n = 3/23, 1 IDH1 R132C, 1 IDH2 R140Q and 1 IDH2 R172K). Time from diagnosis till relapse was not proven to be significantly longer in cases where IDH1/2 was undetectable at relapse compared to cases with persistent IDH1/2 mutation at relapse [median 7.4 month (range: 2.2–11.4 month) versus 8.6 month (range: 0.83–57.2 month) respectively, p = 0.65]. Interestingly in three (IDH1 R132H: n = 1; IDH2 R140Q: n = 2) out of 93 IDH1/2 negative AML cases where diagnosis and relapse samples were both available, IDH1/2 mutations appeared only at relapse. These cases were re-evaluated by the more sensitive ddPCR method at diagnosis and VAF (0–0.23%) was under the detection limit of HRM and/or allele specific PCR in each case.

The applicability of ddPCR methods
NPM1-positive patients screened by capillary electrophoresis at diagnosis were retrospectively typed with type-A and type-N primers using ddPCR. Out of 200 AML patients (53 samples were not available) 97% (n = 194) was proved to be NPM1 type-A or type-N and not more than 3% (n = 6) could not be detected with type-N primer [14] (S3 Table). IDH1 or IDH2 mutations were screened by HRM and allele-specific PCR (S4 Table). Out of the 68 IDH1 positive AML patients 39% (n = 27) was IDH1 R132C, 46% (n = 30) IDH1 R132H, 15% (n = 11) IDH1 R132G/L/S/P. In case of IDH2 positive AML, 81% (n = 76) harbored IDH2 R140Q and 19% (n = 18) IDH2 R172K. In our patient cohort, 93% (151/162) IDH1/2 mutation positive patients carried an IDH1/2 variant detectable with ddPCR. Interestingly, IDH1 R132H was associated with NPM1 type-A mutation in 48% (n = 13/27), while other IDH1 R132 codon mutants and IDH2 R140Q co-occurred with NPM1 type-A mutation in 70% (n = 33/47; p = 0.08; S5 Table).

NPM1 MRD monitoring
The LoD for NPM1 type-A ddPCR was lower than type-N ddPCR both in DNA and RNA settings (S6 Table). NPM1 mutant VAF values in DNA and NPM1 mutant expression levels in
RNA were considered as MRD negative if below 0.01% (type-A) or below 0.05% (type-N). In case of NPM1, 174 NPM1 positive cases reached MLFS after induction, MRD monitoring could not be performed in 5 cases due to technical limitations (NPM1 mutation could not be detected by type-A or type-N primers), and in 53 cases due to non-available DNA. Basic characteristics such as gender, age at diagnosis, induction therapy, HSCT, and outcome (death in aplasia or in indeterminate cause, remission, relapse, cytogenetic and molecular genetic data) of NPM1 positive and MRD monitored patients were included in S3 Table. In 116 NPM1 MRD monitored patients, NPM1 mutant VAF was reduced below 2.5% in all patients in MLFS after induction.

We examined the OS and RFS of 90 AML patients who have NPM1 type-A mutation and further 26 patients who were monitored with type-N NPM1 ddPCR from DNA. The median NPM1 VAF at diagnosis was 45.7% (range: 11.5–49.3%), while after induction therapy was 0.06% (range: 0–2.5%). Out of the 90 patients with type-A NPM1, 35 patients were MRD-negative, and 55 MRD-positive. Favorable outcome measures were observed in MRD negative compared to MRD positive patients for NPM1 type-A (24-month OS: 50.2±8.9% for negative versus 27.7±6.5% for positive, p = 0.010; and 24-month RFS: 40.2±8.6% versus 15.8±5.1% p = 0.009, S1 Fig). MRD-positive patients were further divided into MRD-low and MRD-high burden subgroups. In our patient cohort, NPM1 VAF 0.2% was considered as the limit to discriminate between low and high burden: 28 patients were classified into the MRD low category (ranging from 0.01 to 0.2% NPM1 mutant allele burden), and 27 patients in the MRD high category (above 0.2%). As expected even within the MRD positive subgroup, high allele burden cases showed a tendency to more adverse outcome measures (24-month OS: 40.6±10.3% for low versus 16.1±7.4% for high MRD, p = 0.088; and 24-month RFS: 19.4±7.8% versus 12 ±6.5%; p = 0.107, S1 Fig). Analyses were also performed for NPM1 aggregate types-A and -N, and similar results were obtained, (24-month OS: 58.5±7.5% for negative versus 39.3±6.2% for positive, p = 0.029; and 24-month RFS: 48.3±7.5% versus 27.8±5.6%, p = 0.019, Fig 3). The difference did not reach the level of significance within the MRD positive group (24-month OS: 47.6±9.4% for low versus 32.6±8.0% for high, p = 0.250; and 24-month RFS: 29.3±8.2% versus 26.5±7.6%, p = 0.372, Fig 3). NPM1 type-A and -N MRD positivity proved to be an independent risk factor in multivariate analysis beside age at diagnosis, cytogenetics and FLT3-ITD allele burden, and white blood cell (WBC) count above 100.000 per microliter at diagnosis (OS: HR 2.16 95%CI 1.25–3.74, p = 0.006; RFS: HR 2.21 95%CI 1.32–3.68, p = 0.002) (Table 1).

Fig 3. Probability of overall survival and relapse free survival according to NPM1 MRD after induction. On both panels (A: overall survival; B: relapse free survival), the outcome of NPM1 MRD-negative (MRDneg VAF<0.01–0.05% depending on NPM1 mutation type) and MRD-positive (MRDpos VAF>0.01–0.05%) subgroups are shown with the associated p1 value. The NPM1 MRD-positive subgroup was further divided in MRD low-positive (MRDlow VAF = 0.01–0.2%) and MRD high-positive (MRDhigh VAF > 0.2%) subgroups, and compared with p2 values.

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High allelic ratio of FLT3-ITD at diagnosis (categorized according to the ELN 2017 risk stratification) is a well-documented adverse risk factor in AML. In the favorable subgroup of mutated NPM1 without FLT3-ITD (FLT3-ITD\textsuperscript{neg}) or with low allelic ratio (FLT3-ITD\textsuperscript{low}), the presence NPM1 MRD provided a valuable prognostic biomarker (NPM1 MRD\textsuperscript{neg} versus MRD\textsuperscript{pos} 24-month OS: 66.7±8.6% versus 42.9±6.7%, p = 0.010; RFS: 60±8.9% versus 31.1±6.2%, p = 0.006). NPM1 MRD did not influence survival in the FLT3-ITD\textsuperscript{high} subgroup. NPM1 MRD negative and FLT3-ITD high allele burden resulted similar survival measures to NPM1 MRD positive patients (Fig 4).

Out of the 38 patients who underwent allo-HSCT for MRD monitoring with the NPM1 mutation type-A (n = 27) and type-N (n = 11), pre-HSCT sample was available in 32 (24 type-A and 8 type-N). NPM1 MRD negativity before allo-HSCT proved to be favorable prognostic factor, OS after HSCT was significantly longer in MRD negative compared to positive patients (24-month OS MRD\textsuperscript{neg}: 74.7±9.8% versus MRD\textsuperscript{pos}: 16.2±14.6%, p = 0.012; Fig 5).

Similarly to genomic DNA two or three log reduction was observable in mutant NPM1 RNA expression (NPM1/ABL1, n = 39 patients) [at diagnosis: median 610.8% (range: 124.3–2882.4%), after induction: 1.0% (range: 0–398%)]. Despite the low number of RNA samples, high mutant NPM1 expression after induction correlated with unfavorable outcome (24-month OS mutant NPM1 expression <1%: 55.2±12.9% versus mutant NPM1 expression >1% 20.0±11.9%, p = 0.005; and 24-month RFS: 51.6%±12.5% versus 12±7.9% respectively;

### Table 1. Multivariate analysis of NPM1 MRD status after induction.

|                          | Overall Survival (n = 116) | Relapse Free Survival (n = 116) |
|--------------------------|----------------------------|---------------------------------|
|                          | Hazard ratio (95% CI)      | P                               |
| NPM1 MRD positivity\textsuperscript{a} | 2.16 (1.25–3.74)          | 0.006                           |
| Age (per year)           | 1.02 (1.00–1.04)           | 0.019                           |
| Cytogenetics\textsuperscript{b} | 1.50 (0.86–2.63)          | 0.155                           |
| FLT3-ITD\textsuperscript{c} | 1.75 (1.19–2.56)           | 0.004                           |
| WBC >100.000/μL          | 0.88 (0.50–1.56)           | 0.656                           |

\textsuperscript{a}NPM1 MRD positivity was defined as VAF>0.01–0.05% depending on mutation type.

\textsuperscript{b}Cytogenetics coded as normal karyotype (reference), other intermediate and adverse risk.

\textsuperscript{c}FLT3-ITD coded in three categories as wild type (reference), low and high allelic ratio.

Abbreviations: 95% CI: 95% confidence interval; FLT3-ITD: fms-like tyrosine kinase 3 –internal tandem duplication; MRD: measurable residual disease; NPM1: nucleophosmin1; WBC: white blood cell count at diagnosis.
We investigated parallel RNA and DNA-based NPM1mut ddPCR methods from 39 samples after first induction therapy from 39 patient who have both DNA and RNA samples. The assay sensitivity proved to be higher on RNA samples. Altogether 46% of the RNA samples that displayed NPM1mut expression (median: 0.1%; range: 0.01–5.1%) were detected as negative in the matching DNA samples (<0.01%). RNA assay (NPM1mut expression) proved to be more sensitive (median: 1.3-log; range: 0.0–2.78-log) compared to DNA assay (NPM1mut VAF) in samples with concomitant positivity both on RNA and DNA level (S2 Fig).

**IDH1/2 MRD monitoring**

The LoB for IDH1/2 mutation detection was 0.06–0.08% and the LoD was 0.09–0.12% (S6 Table). In general, VAF below 0.2% for each IDH1/2 form was considered as negative. In case of 90 IDH1/2 positive patients in MLFS after induction, MRD monitoring could not be performed in 8 cases with IDH1R132G/L/S/P/ and in 20 cases with lacking DNA samples. Basic characteristics of IDH1/2 positive and MRD monitored patients were included in S4 Table. We observed that IDH1/2 VAF in morphologic leukemia free state was not reduced below 2.5% in 15 out of 62 cases (24%, 10 IDH2 R140Q, 3 IDH2 R172K, 1 IDH1 R132H and 1 IDH1 R132C). Seven cases were NPM1 positive (6 IDH2 R140Q and 1 IDH1 R132H) at diagnosis but NPM1 mutational burden was reduced below 2.5% in remission. Regarding the outcome of patients with persisting IDH1/2 mutation: 9 patients relapsed and subsequently died, 2 patients alive after HSCT, 3 patients alive in complete remission after 12 months follow up and 1 patient died without relapse.

In our analyses, the survival of IDH1 or IDH2 MRD-negative patients was significantly better than that of MRD-positive patients (24-month OS MRDneg: 62.5±9% versus MRDpos: 41.3±9.2%, p = 0.003; 24-month RFS: 45.0±9.3% versus 38.8±9.6% respectively, p = 0.027, Fig 6).
In multivariate analysis, IDH1/2 MRD positivity was proved to be an independent risk factor for survival besides age, cytogenetics, FLT3-ITD, NPM1 and WBC (OS: HR: 2.81 95%CI: 1.09–7.23, p = 0.032, RFS: HR: 2.80 95%CI: 1.15–6.82, p = 0.023, Table 2).

In allo-HSCT cases, pre-HSCT samples were available in 21 out of 22 patients (10 IDH1 and 11 IDH2). IDH1/2 MRD negativity (VAF < 0.2%) before allo-HSCT did not reach statistical significance (24-month OS MRD neg: 92.3 ± 7.4% versus MRD pos 68.6 ± 18.6%, p = 0.149).

IDH1/2 MRD below 2.5% influenced significantly survival (24-month OS: MRD < 2.5% 87.8 ± 8.1% versus MRD > 2.5% 50.0±25.0% before allo HSCT, p = 0.015; Fig 7).

**Discussion**

The serial acquisition of somatic mutations in myeloid clone(s) was described as the multistep pathogenesis of AML. Several lines of evidence prove that NPM1 mutations are responsible for the definitive acute leukemic transformation, therefore considered as leukemia founder mutations: (i) NPM1 mutations are completely absent in the population without hematological malignancies even at a higher age [22–24]; (ii) NPM1 mutations cannot be detected in AML patients months or years before the manifestation of AML [25, 26]; (iii) NPM1 mutations occur rather rarely (approximately 2–3%) in preleukemic myeloid malignancies such as myelodysplastic syndrome (MDS) or in myelodysplastic/myeloproliferative neoplasm.

![Fig 6. Overall survival and relapse free survival according to IDH1/2 MRD after induction.](https://doi.org/10.1371/journal.pone.0253386.g006)

Table 2. Multivariate analysis of IDH1/2 MRD status after induction.

|                         | Overall Survival (n = 62) | Relapse Free Survival (n = 62) |
|-------------------------|---------------------------|-------------------------------|
|                         | Hazard ratio (95% CI)     | P                             | Hazard ratio (95% CI)     | P                             |
| IDH1/2 MRD positivitya  | 2.81 (1.09–7.23)          | 0.032                         | 2.80 (1.15–6.82)          | 0.023                         |
| Age per year            | 1.03 (0.99–1.06)          | 0.126                         | 1.02 (0.99–1.05)          | 0.256                         |
| Cytogeneticsb           | 1.98 (0.90–4.33)          | 0.089                         | 2.36 (1.07–5.21)          | 0.034                         |
| FLT3-ITDc               | 1.00 (0.42–2.38)          | 0.994                         | 1.06 (0.49–2.28)          | 0.889                         |
| NPM1                    | 1.62 (0.57–4.58)          | 0.364                         | 2.26 (0.82–6.25)          | 0.115                         |
| WBC >100.000/ul         | 1.17 (0.49–2.78)          | 0.727                         | 1.04 (0.45–2.43)          | 0.922                         |

*a*IDH1/2 MRD positivity was defined as VAF>0.2%.

bCytogenetics coded as normal karyotype (reference), other intermediate and adverse risk.

cFLT3-ITD coded in three categories as wild type (reference), low and high allelic ratio.

Abbreviations: 95%CI: 95% confidence interval; IDH1/2: isocitrate dehydrogenase 1/2; FLT3-ITD: fms-like tyrosine kinase 3–internal tandem duplication; MRD: measurable residual disease; NPM1: nucleophosmin1; WBC: white blood cell count at diagnosis.

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(MDS/MPN), which are mainly characterized by the progression to overt AML [27]; (iv) NPM1 mutations were not present in preleukemic hematopoietic stem cells [28, 29]. Our observation that NPM1 VAF decreased below 2.5% in all cases with morphologic leukemia-free state, also proved that NPM1 mutations do not occur in the preleukemic state. (v) A further proof that NPM1 mutations harbor leukemia-initiating properties is the high reappearance rate of NPM1 in relapse, which was demonstrated as high as 86–100% in several clinical observations [30–39]. The long observational period in our study allowed us to detect late AML relapses. In line with previous studies less than 10% of our NPM1 mutation positive cases relapsed as wild type NPM1 AML. The loss of NPM1 mutation in our patient cohort was not associated with longer remission before relapse, which was suggested by several previous studies [31, 32]. Although our study did not investigate the spectrum of preleukemic mutations, the persistence of IDH2 R140Q mutation was observed in a single case with NPM1-mutation loss relapse 13 month after diagnosis.

Contradictory data exist in the literature, whether IDH1 and IDH2 mutations are preleukemic or AML founder mutations. Several studies suggest IDH1/2 mutations as epigenetic modifiers as preleukemic events. (i) In large scale populational screening studies for clonal hematopoeisis of indeterminate potential (CHIP) mutations, IDH2 R140 mutations were extremely rarely detected in elderly individuals (IDH2 R140Q/W: 0.014%, four out of 29562 individuals) [22]. (ii) IDH1 and IDH2 mutations were detectable as premalignant, high-risk gene mutations years before the diagnosis of AML, but not in age-matched controls (8%; n = 15/188; three IDH1 R132C/H/G and 12 IDH2 R140 positive individuals with a median of 7 years before AML diagnosis) [26]. (iii) IDH1 and IDH2 mutations are also rarely present in preleukemic myeloid malignancies: 0.8–4% in chronic phase MPN, 4–14% in MDS, but its frequency increases up to 20–25% in blast phase transformation [40–43]. (iv) IDH1 and IDH2 mutations were detectable in preleukemic hematopoietic stem cells [28, 29]. The comparison of VAF values suggested that IDH1 and IDH2 mutations were more likely to develop before NPM1 mutations [6]. The persistence of IDH1/2 mutations (especially IDH2 R140Q) in remission was observed in 7–39% of AML cases in the literature, [19, 44–46] which is in line with our study (24% of IDH1 or IDH2 mutations were detectable in complete remission with a higher than 2.5% VAF, 67% of persisting mutations was IDH2 R140Q). The high mutational load in remission is a direct proof of preleukemic origin of the somatic mutation. This phenomenon in case IDH1/2 mutations is not as frequent as in case of DNMT3A, TET2, ASXL1 gene mutations, where reported rates vary between 51–82% [2, 47–49], (v) At relapse both IDH1/2 gene mutations showed a relatively high stability (86–88% reported in publications, 87% in our study) similar that of NPM1 mutation [31]. (vi) In IDH1/2 mutation negative AML, the emergence of IDH1 or IDH2 mutations at relapse was observed in 10% in our study,
which suggests the subclonal, late origin of these mutations. Interestingly, there is a usual mutation order in AML pathogenesis, but some mutations might appear both early and late events [31, 50].

A recent meta-analysis proved that lower MRD was consistently associated with improved outcome independently from applied method, sample source or sampling time of the assessment [51]. Regarding molecular genetic detection techniques, like quantitative PCR, digital PCR and next generation sequencing are extensively applied for MRD detection. High assay precision and reproducibility make ddPCR particularly suitable for MRD monitoring, which was reported in connection with several oncohematological drivers [52–56]. Individual assay designs make the quantification of multiple \( NPM1 \) mutations challenging, but the application of degenerated primers allows the simultaneous detection of multiple \( NPM1 \) mutations affecting the same localization (c.860_863dupNNNN) [14]. Our data and other studies also supported, that less than 5% of \( NPM1 \) mutations affects nucleotides at different positions [11, 38, 57].

Although consensus exists about the importance of \( NPM1 \) MRD; broad range of heterogeneity was displayed concerning thresholds discriminating between low- and high-risk MRD. Studies comparing mutant \( NPM1 \) transcript levels parallel in bone marrow (BM) and peripheral blood (PB) samples identified strong correlation, but an average of 1-log higher sensitivity in BM [21, 34, 38, 57–61]. In line with this observation, 3-log reduction of \( NPM1^\text{mut}/ABL1 \) transcript level was pointed as favorable prognostic indicator in BM, [21, 59, 60] but 4-log reduction was required in PB after induction therapy [57, 58]. In our study, bone marrow samples were processed. As \( NPM1^\text{mut} \) expression is highly abundant, greater sensitivity (median: 1.3 log, range: 0.2–2.78 log in our study) was achieved on RNA level than on DNA. \( NPM1^\text{mut} \) RNA expression level detection for MRD monitoring is recommended in the literature [13, 38]. Shayegi et al. investigated that 1% \( NPM1^\text{mut}/ABL1 \) expression corresponds to 0.016% \( NPM1^\text{mut} \) VAF or 1 in 32000 cells (1.8 log difference between RNA and DNA levels) [60].

These data suggested that \( NPM1^\text{mut} \) MRD screening should be performed on RNA expression, but in case of RNA unavailability, highly sensitive DNA methods can substitute. The applied cut-off for MRD negativity in our study (\( NPM1 \) type-A: 0.01% and type-N: 0.05% on DNA level) corresponds approximately to 1% \( NPM1^\text{mut}/ABL1 \) expression level. We were unable to test large number of RNA samples, which is a major limitation of our retrospective study. Ivey et al. [57] demonstrated that RNA-MRD positivity in PB after induction (2 cycles) corresponded to higher cumulative incidence of relapse (MRC17 trial 3-year CIR: 82% versus 30%), similarly Balsat et al. [58] (ALFA-0702 trial: 2-year CIR: 55% versus 21%); Hubmann et al. [62] less than 3-log-reduction in BM RNA-MRD (AMLCG 1999, 2004 and 2008 trial: 2-year CIR 77.8% versus 26.4%), Kapp-Schwoerer et al. [34] less than 3-log\(_{10}\) BM or PB RNA-MRD (AMLSG 09–09 trial 4-year CIR BM: 60% versus 28.5%; PB: 62.5% versus 33.9%). On the DNA level, we also observed that MRD positivity (less than 3-log reduction) was associated with adverse outcome, and DNA-MRD after induction therapy is capable to identify high-risk \( NPM1^\text{mut} \) patients.

The co-occurrence with \( FLT3-ITD \) was recognized as an adverse factor in \( NPM1 \) mutant AML, due to the highly proliferative nature of the leukemic clone with ITD [38, 63]. Although \( NPM1 \) mutation was referred as favorable or intermediate ELN prognostic categories depending on the presence of \( FLT3-ITD \) with high mutational load [1]. Recently, the reclassification of ELN prognostic criteria identified high \( FLT3-ITD \) load as adverse risk irrespective of \( NPM1 \) mutation status [64]. Allogeneic HSCT in first complete remission is not recommended in favorable risk AML, on the other hand relapsed \( NPM1 \)-positive cases have adverse outcome [65]. We observed that the measurement of \( NPM1 \) MRD was capable to identify high risk patients even in the favorable risk \( NPM1 \) positive AML without high ITD load. \( NPM1 \) MRD
negativity ($NPM1^{mut}$ VAF <0.01–0.05% after induction) with high $FLT3$-ITD allele burden at diagnosis showed similarly adverse survival to $NPM1$ MRD positive patients.

Molecular MRD measurements serve not only prognostic, but may influence therapy. In case of persistent MRD, HSCT consolidation improved survival over chemotherapy [66]. In ELN 2017 favorable risk $NPM1^{mut}$ AML subgroup, molecular failure (defined as $NPM1^{mut}$/ $ABL1$ >0.05% after consolidation or $NPM1^{mut}$ reappearance after molecular response; which affected 40% of $NPM1^{mut}$ cases) served as indication for allogeneic HSCT in first complete remission. MRD-guided approach involving early intervention resulted in improved outcome (two-year OS: 85% for HSCT-treated patients with molecular failure and 39% for patients with hematological relapse) [67]. For elderly or unfit patients, azacitidine was reported to prevent or delay hematological relapse in MRD-positive AML [68].

Our data investigating pre HSCT $NPM1^{mut}$ MRD are in good concordance with other studies with similar MRD time-point assessment: pre HSCT MRD negativity predicts favorable outcome after HSCT [21, 66, 69–71]. Detection of MRD-positivity before HSCT guide therapeutic choices during conditioning and graft versus host disease prevention, e. g. preferably T-cell repleted versus T-cell depleted transplant [21]; preferably myeloablative versus reduced intensity conditioning [72]. MRD measurements can even guide targeted $FLT3$-inhibitor therapy identifying patients who benefit mostly [73].

The role of MRD-monitoring is well-documented in case of $NPM1$, but data are scarce about $IDH1$ and $IDH2$ mutations. We applied BioRad-designed mutation detection reagents on BioRad QX200 Droplet Digital PCR System, but interestingly we were not able to reach as high sensitivity as in case of $NPM1$. Similar technical limits (LoD: 0.2%) were reported in a previous study applying the same detection [19]. Our data also supported the preleukemic nature of $IDH1/2$ mutations, but the persistence of $IDH1/2$ mutations (VAF>2.5%) in complete remission was associated with adverse outcome, higher chance of relapse or the development of myelodysplasia [19, 44]. The presence of a preleukemic clone in morphologic leukemia-free remission was generally reported to associate with inferior survival compared to patients without persisting oncogenic mutations [74, 75]. On the other hand, persistent $DNMT3A$, $TET2$, $ASXL1$ mutations were not connected with higher relapse rate and several reports described long-term remission even with high VAF [2, 47–49]. The frequency of persistent $IDH1/2$ mutations in remission was reported as high as 7–39% depending on the VAF cut-off (1–5%) or on the applied chemotherapy [2, 19, 44, 45], which was similar to our observation (24%). In line with previous publications [19, 44, 45], our data also indicated that persisting $IDH1/2$ mutations in remission were associated with adverse prognostic impact. Currently no guidelines exist whether pre-emptive therapeutic interventions (such as HSCT or $IDH1/2$ inhibitors) could reduce relapse rate or improve survival in case of persisting $IDH1/2$ mutations in remission. The combination of $IDH1$ or $IDH2$ inhibitors with intensive chemotherapy in newly diagnosed AML might improve mutation clearance, although no comparative data exist with or without the inhibitors [76].

In summary, we investigated a considerably large number of AML patients systematically over a long time, the limitation of our study is the retrospective study design and the heterogeneous treatment protocols applied during the observational period. Our results support that $NPM1$ MRD even at DNA level is a reliable prognostic factor. On the other hand, $IDH1/2$ mutations may represent pre-leukemic, founder or subclonal drivers, still $IDH1/2$ MRD may also identify high risk AML. As MRD represents a biological continuum, special detailed guidelines are required to establish proper thresholds for the initiation of pre-emptive therapies.
Supporting information

S1 Table. Primers and probes used in NPM1 ddPCR.
(XLSX)

S2 Table. Cytogenetic and molecular genetic characteristics of 916 AML patients. Abbreviations for S2–S5 Tables: DNR&AraC: standard daunorubicin&cytarabine regimen; FLT3-ITD: fms-like tyrosine kinase internal tandem duplication, FLT3-TKD: fms-like tyrosine kinase tyrosine kinase domain, HSCT: hematopoietic stem cell transplantation, IDH: isocitrate dehydrogenase, MLFS: morphologic leukemia-free state, MRD: measurable residual disease, NPM1 mutation type not available*: patients with palliative treatment or with missing DNA samples were not further evaluated for NPM1 mutation type.
(XLSX)

S3 Table. Cytogenetic and molecular genetic characteristics of NPM1 positive AML patients.
(XLSX)

S4 Table. Cytogenetic and molecular genetic characteristics of IDH1 and IDH2 positive AML patients.
(XLSX)

S5 Table. Baseline characteristics of NPM1 and IDH1/2 positive AML patients.
(XLSX)

S6 Table. Descriptives of the applied ddPCR methods. Abbreviations: LoB: limit of blank, LoD: limit of detection.
(XLSX)

S1 Fig. Probability of overall survival and relapse free survival according to NPM1 type-A MRD after induction. On both panels (A: overall survival; B: relapse free survival), the outcome of NPM1 type-A MRD-negative (MRD\text{neg} VAF<0.01%) and MRD-positive (MRD\text{pos} VAF>0.01%) subgroups are shown with the associated p1 value. The NPM1 type-A MRD-positive subgroup was further divided in MRD low-positive (MRD\text{low} VAF = 0.01–0.2%) and MRD high-positive (MRD\text{high} VAF > 0.2%) subgroups, and compared with p2 values.
(TIF)

S2 Fig. Comparison of DNA and RNA based NPM1 mutation MRD detection after induction. DNA based method describes the variant allele frequencies of mutant NPM1 (NMP1\text{mut}/GAPDH ratio), while RNA method showed the NPM1 RNA mutation expression (NPM1\text{mut}/ABL1). RNA samples that displayed NPM1\text{mut} expression and VAF negativity in the are marked with the grey continuous lines (18 samples, 46%). Samples with at least 0.5 log higher RNA expression level with detectable mutant NPM1 allele frequency on DNA level are shown with black dashed lines (19 samples, 49%). Only two samples (black continuous lines, 5%) showed equivalent NPM1\text{mut} RNA expression and DNA allele burden.
(TIF)

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References

1. Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017; 129(4):424–47. https://doi.org/10.1182/blood-2016-08-733196 PMID: 27895058

2. Jongen-Lavrencic M, Grob T, Hanekamp D, Kavelaars FG, Al Hnai A, Zeilemaker A, et al. Molecular Minimal Residual Disease in Acute Myeloid Leukemia. The New England journal of medicine. 2018; 378(13):1189–99. https://doi.org/10.1056/NEJMoa1716863 PMID: 29601269

3. Schuurhuis GJ, Hauser M, Freeman S, Béné MC, Buccisano F, Cloos J, et al. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. Blood. 2018; 131(12):1275–91. https://doi.org/10.1182/blood-2017-09-801498 PMID: 29330221

4. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. The New England journal of medicine. 2015; 373(12):1136–52. https://doi.org/10.1056/NEJMra1406184 PMID: 26376137

5. Falini B, Brunetti L, Sportoletti P, Martelli MP. NPM1-mutated acute myeloid leukemia: from bench to bedside. Blood. 2020; 136(15):1707–21. https://doi.org/10.1182/blood.2019004226 PMID: 32609823

6. Papamannu E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. The New England journal of medicine. 2016; 374(23):2209–21. https://doi.org/10.1056/NEJMoa1516192 PMID: 27276561

7. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer cell. 2010; 18(6):553–67. https://doi.org/10.1016/j.ccr.2010.11.015 PMID: 21130701

8. Grimwade D, Ivey A, Huntly BJ. Molecular landscape of acute myeloid leukemia in younger adults and its clinical relevance. Blood. 2016; 127(1):29–41. https://doi.org/10.1182/blood-2015-07-604496 PMID: 26660431

9. Koszarska M, Bors A, Feczko A, Meggyesi N, Batai A, Csomor J, et al. Type and location of isocitrate dehydrogenase mutations influence clinical characteristics and disease outcome of acute myeloid leukemia. Leukemia & lymphoma. 2013; 54(5):1028–35. https://doi.org/10.3109/10428194.2012.736981 PMID: 23099322

10. Kövy P, Kozma A, Bors A, Meggyesi N, Ádám E, Borsy A, et al. Új terápiás célpont akut myeloid leukémiaiban: izocitrát dehidrogenáz 1 és 2 mutációi. Hematológia Transzfuziológia. 2019; 152:142–7.
11. Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood. 2006; 107(10):4011–20. https://doi.org/10.1182/blood-2005-08-3167 PMID: 16455956

12. Chou WC, Tang JL, Wu SJ, Tsay W, Yao M, Huang SY, et al. Clinical implications of minimal residual disease monitoring by quantitative polymerase chain reaction in acute myeloid leukemia patients bearing nucleophosmin (NPM1) mutations. Leukemia. 2007; 21(5):998–1004. https://doi.org/10.1038/sj.leu.2404637 PMID: 17361227

13. Gorello P, Cazzaniga G, Alberti F, Dell’Oro MG, Gottardi E, Specchia G, et al. Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. Leukemia. 2006; 20(6):1103–8. https://doi.org/10.1038/sj.leu.2404144 PMID: 16511444

14. Mencia-Trinant N, Hu Y, Alas MA, Ali F, Wouters BJ, Lee S, et al. Minimal Residual Disease Monitoring of Acute Myeloid Leukemia by Massively Multiplex Digital PCR in Patients with NPM1 Mutations. The Journal of molecular diagnostics: JMD. 2017; 19(4):537–48. https://doi.org/10.1016/j.jmoldx.2017.03.005 PMID: 28525762

15. Waterhouse M, Pfeifer D, Duque-Afonso J, Follo M, Duyster J, Depner M, et al. Droplet digital PCR for the simultaneous analysis of minimal residual disease and hematopoietic chimerism after allogeneic cell transplantation. Clin Chem Lab Med. 2019; 57(5):641–7. https://doi.org/10.1515/cclmd-2018-0827 PMID: 30457973

16. Alizadeh M, Bernard M, Danic B, Dauriac C, Birebent B, Lapart C, et al. Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. Blood. 2002; 99(12):4618–25. https://doi.org/10.1182/blood.v99.12.4618 PMID: 12038896

17. Beillard E, Pallisgaard N, van der Velden VH, Bi W, Dee R, van der Schoot E, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using ‘real-time’ quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR)—a Europe against cancer program. Leukemia. 2003; 17(12):2474–86. https://doi.org/10.1038/sj.leu.2403136 PMID: 14562124

18. Cross NC, White HE, Colomer D, Ehrenconra H, Foroni L, Gottardi E, et al. Laboratory recommendations for scoring deep molecular responses following treatment for chronic myeloid leukemia. Leukemia. 2015; 29(5):999–1003. https://doi.org/10.1038/leu.2015.29 PMID: 25652737

19. Ferret Y, Boissel N, Helevaut N, Madic J, Nibourel O, Marceau-Renaut A, et al. Clinical relevance of IDH1/2 mutant allele burden during follow-up in acute myeloid leukemia. A study by the French ALFA group. Haematologica. 2018; 103(5):822–9. https://doi.org/10.3324/haematol.2017.183525 PMID: 29472349

20. Topić E, Nikolac N, Panteghini M, Theodorsson E, Salvagno GL, Miller M, et al. How to assess the quality of your analytical method? Clin Chem Lab Med. 2015; 53(11):1707–18. https://doi.org/10.1515/cclmd-2015-0869 PMID: 26408611

21. Dillon R, Hills R, Freeman S, Potter N, Jovanovic J, Ivey A, et al. Molecular MRD status and outcome after transplantation in NPM1-mutated AML. Blood. 2020; 135(9):680–8. https://doi.org/10.1182/blood.2019002959 PMID: 31932839

22. Genovesi G, Kähler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoun SF, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. The New England journal of medicine. 2014; 371(26):2477–87. https://doi.org/10.1056/NEJMoa1409405 PMID: 25426838

23. Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG, et al. Age-related clonal hematopoiesis associated with adverse outcomes. The New England journal of medicine. 2014; 371(26):2488–98. https://doi.org/10.1056/NEJMoa1408617 PMID: 25426837

24. Xie M, Lu C, Wang J, McLellan MD, Johnson KJ, Wendl MC, et al. Age-related mutations associated with clonal hematopoetic expansion and malignancies. Nat Med. 2014; 20(12):1472–8. https://doi.org/10.1038/nm.3733 PMID: 25326804

25. Abelson S, Collord G, Ng SWK, Weissbrod O, Mendelson Cohen N, Niemeyer E, et al. Prediction of acute myeloid leukemia risk in healthy individuals. Nature. 2018; 559(7714):400–4. https://doi.org/10.1038/s41586-018-0317-6 PMID: 29988082

26. Desai P, Mencia-Trinchant N, Savenkov O, Simon MS, Cheang G, Lee S, et al. Somatic mutations precede acute myeloid leukemia years before diagnosis. Nat Med. 2018; 24(7):1015–23. https://doi.org/10.1038/s41591-018-0081-z PMID: 29988143

27. Forgieri F, Nasillo V, Paolini A, Bettelli F, Pioli V, Giusti D, et al. NPM1-Mutated Myeloid Neoplasms with <20% Blasts: A Really Distinct Clinico-Pathologic Entity? Int J Mol Sci. 2020; 21(23).

28. Corces-Zimmerman MR, Hong WJ, Weissman IL, Medeiros BC, Majetich R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. Proc Natl Acad Sci U S A. 2014; 111(7):2548–53. https://doi.org/10.1073/pnas.1324297111 PMID: 24550281
29. Shlush LI, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature. 2014; 506(7488):328–33. https://doi.org/10.1038/nature13038 PMID: 24522528

30. Chou WC, Tang JL, Lin LI, Yao M, Tsay W, Chen CY, et al. Nucleophosmin mutations in de novo acute myeloid leukemia: the age-dependent incidences and the stability during disease evolution. Cancer Res. 2006; 66(6):3310–6. https://doi.org/10.1158/0008-5472.CAN-05-4316 PMID: 16540685

31. Cocciardi S, Dolnik A, Kapp-Schworer S, Rücker FG, Lux S, Blätte TJ, et al. Clonal evolution patterns in acute myeloid leukemia with NPM1 mutation. Nat Commun. 2019; 10(1):2031. https://doi.org/10.1038/s41467-019-09745-2 PMID: 31048683

32. Hölllein A, Meggendorfer M, Dicker F, Jeromin S, Nadarajah N, Kern W, et al. NPM1 mutated AML can relapse with wild-type NPM1: persistent clonal hematopoiesis can drive relapse. Blood advances. 2018; 2(22):3118–25. https://doi.org/10.1182/bloodadvances.2018023432 PMID: 30455361

33. Jain P, Kantarjian H, Patel K, Faderl S, Garcia-Manero G, Benjamin O, et al. Mutated NPM1 in patients with acute myeloid leukemia in remission and relapse. Leukemia & lymphoma. 2014; 55(6):1337–44. https://doi.org/10.3109/10428194.2013.840776 PMID: 24004182

34. Kapp-Schworer S, Weber D, Corbacioglu A, Gaidzik VI, Paschka P, Krönke J, et al. Impact of gemtuzumab ozogamicin on MRD and relapse risk in patients with NPM1-mutated AML: results from the AMLSG 09–09 trial. Blood. 2020; 136(26):3041–50. https://doi.org/10.1182/blood.2020005998 PMID: 33367545

35. Krönke J, Bullinger L, Teleaun V, Tschürtz F, Gaidzik VI, Kühn MW, et al. Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. Blood. 2013; 122(1):100–8. https://doi.org/10.1182/blood-2013-01-479188 PMID: 23704090

36. Meloni G, Mancini M, Gianfelici V, Martelli MP, Foa R, Falini B. Late relapse of acute myeloid leukemia with mutated NPM1 after eight years: evidence of NPM1 mutation stability. Haematologica. 94. Italy 2009. p. 298–300. https://doi.org/10.3324/haematol.2008.000059 PMID: 19181793

37. Papadalci C, Dufour A, Seibl M, Schneider S, Bohlander SK, Zellmeier E, et al. Monitoring minimal residual disease in acute myeloid leukemia with NPM1 mutations by quantitative PCR: clonal evolution is a limiting factor. Br J Haematol. 2009; 144(4):517–23. https://doi.org/10.1111/j.1365-2141.2008.07488.x PMID: 19055671

38. Schnittert S, Kern W, Tschulik C, Weiss T, Dicker F, Falini B, et al. Minimal residual disease levels assessed by NPM1 mutation-specific RQ-PCR provide important prognostic information in AML. Blood. 2009; 114(11):2220–31. https://doi.org/10.1182/blood-2009-03-213389 PMID: 19587375

39. Suzuki T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R, et al. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood. 2005; 106(6):2584–61. https://doi.org/10.1182/blood-2005-04-1733 PMID: 15994285

40. Lin CC, Hou HA, Chou WC, Kuo YY, Liu CY, Chen CY, et al. IDH mutations are closely associated with mutations of DNMT3A, ASXL1 and SRSF2 in patients with myelodysplastic syndromes and are stable during disease evolution. Am J Hematol. 2014; 89(2):137–44. https://doi.org/10.1002/ajh.23596 PMID: 24115220

41. Medeiros BC, Fathi AT, DiNardo CD, Pollyea DA, Chan SM, Swords R. Isocitrate dehydrogenase mutations in myeloid malignancies. Leukemia. 2017; 31(2):272–81. https://doi.org/10.1038/leu.2016.275 PMID: 27721426

42. Tefferi A, Jirima T, Sulai NH, Lasho TL, Finke CM, Knudson RA, et al. IDH mutations in primary myelofibrosis predict leukemic transformation and shortened survival: clinical evidence for leukemogenic collaboration with JAK2V617F. Leukemia. 2012; 26(3):475–80. https://doi.org/10.1038/leu.2011.253 PMID: 21912393

43. Tefferi A, Lasho TL, Abdel-Wahab O, Guglielmelli P, Patel J, Caramazza D, et al. IDH1 and IDH2 mutation studies in 1473 patients with chronic-, fibrotic- or blast-phase essential thrombocytemia, polycythemia vera or myelofibrosis. Leukemia. 2010; 24(7):1302–9. https://doi.org/10.1038/leu.2010.113 PMID: 20508616

44. Debarri H, Lebon D, Roumier C, Cheek M, Marceau-Renaut A, Nibourel O, et al. IDH1/2 but not DNMT3A mutations are suitable targets for minimal residual disease monitoring in acute myeloid leukemia patients: a study by the Acute Leukemia French Association. Oncotarget. 2015; 6(39):42345–53. https://doi.org/10.18632/oncotarget.5645 PMID: 26486081

45. Ok CY, Loghavi S, Sui D, Wei P, Kanagal-Shamanna R, Yin CC, et al. Persistent IDH1/2 mutations in remission can predict relapse in patients with acute myeloid leukemia. Haematologica. 2019; 104(2):305–11. https://doi.org/10.3324/haematol.2018.1911148 PMID: 30171025

46. Wiseman DH, Williams EL, Wilks DP, Sun Leong H, Somerville TD, Dennis MW, et al. Frequent reconstitution of IDH2(R140Q) mutant clonal multilineage hematopoiesis following chemotherapy for acute...
myeloid leukemia. Leukemia. 30 2016. p. 1946–50. https://doi.org/10.1038/leu.2016.93 PMID: 27118404

47. Gaidzik VI, Weber D, Paschka P, Kaumanns A, Krieger S, Corbacioglu A, et al. DNMT3A mutant transcript levels persist in remission and do not predict outcome in patients with acute myeloid leukemia. Leukemia. 2018; 32(1):30-7. https://doi.org/10.1038/leu.2017.200 PMID: 28643785

48. Plbn GG, Nederby L, Guldberg P, Hansen M, Ebbesen LH, Jensen UB, et al. Persistence of DNMT3A mutations at long-term remission in adult patients with AML. Br J Haematol. 2014; 167(4):478–86. https://doi.org/10.1111/bjh.13062 PMID: 25371149

49. Sun Y, Shen H, Xu T, Yang Z, Qiu H, Sun A, et al. Persistent DNMT3A mutation burden in DNMT3A mutated adult cytogenetically normal acute myeloid leukemia patients in long-term remission. Leuk Res. 2016; 49:102–7. https://doi.org/10.1016/j.leukres.2016.09.001 PMID: 27626217

50. Tuval A, Shlush LI. Evolutionary trajectory of leukemic clones and its clinical implications. Haematologica. 2019; 104(5):872–80. https://doi.org/10.3324/haematol.2018.195289 PMID: 31004016

51. Short NJ, Zhou S, Fu C, Berry DA, Walter RB, Freeman SD, et al. Association of Measurable Residual Disease With Survival Outcomes in Patients With Acute Myeloid Leukemia: A Systematic Review and Meta-analysis. JAMA Oncol. 2020; 6(12):1890–9. https://doi.org/10.1001/jamaoncol.2020.4600 PMID: 33030517

52. Chung HJ, Hur M, Yoon S, Hwang K, Lim HS, Kim H, et al. Performance Evaluation of the QXDx BCR-ABL %IS Droplet Digital PCR Assay. Ann Lab Med. 2020; 40(1):72–5. https://doi.org/10.3343/alm.2020.40.1.72 PMID: 31432643

53. Link-Lenczowska D, Pallisgaard N, Cordua S, Zawada M, Czekalska S, Krochmalczyk D, et al. A comparison of qPCR and ddPCR used for quantification of the JAK2 V617F allele burden in Ph negative MPNs. Ann Hematol. 2018; 97(12):2299–308. https://doi.org/10.1007/s00277-018-3451-1 PMID: 30056580

54. Maier J, Lange T, Cross M, Wildenberger K, Niederwieser D, Franke GN. Optimized Digital Droplet PCR for BCR-ABL. The Journal of molecular diagnostics: JMD. 2019; 21(1):27–37. https://doi.org/10.1016/j.jmoldx.2018.08.012 PMID: 30347270

55. Wang WJ, Zheng CF, Liu Z, Tan YH, Chen XH, Zhao BL, et al. Droplet digital PCR for BCR-ABL(P210) detection of chronic myeloid leukemia: A high sensitive method of the minimal residual disease and disease progression. Eur J Haematol. 2018; 101(3):291–6. https://doi.org/10.1111/ejha.13084 PMID: 29691899

56. Waterhouse M, Follo M, Pfeifer D, von Bubnoff N, Duyster J, Bertz H, et al. Sensitive and accurate quantification of JAK2 V617F mutation in chronic myeloproliferative neoplasms by droplet digital PCR. Ann Hematol. 2016; 95(5):739–44. https://doi.org/10.1007/s00277-016-2623-0 PMID: 26931113

57. Ivey A, Hills RK, Simpson MA, Jovanovic JV, Gilkes A, Grech A, et al. Assessment of Minimal Residual Disease in Standard-Risk AML. The New England journal of medicine. 2016; 374(5):422–33. https://doi.org/10.1056/NEJMoa1507471 PMID: 26789727

58. Balsat M, Renneville A, Thomas X, de Botton S, Marceau A, et al. Postinduction Minimal Residual Disease Predicts Outcome and Benefit From Allogeneic Stem Cell Transplantation in Acute Myeloid Leukemia With NPM1 Mutation: A Study by the Acute Leukemia French Association Group. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2017; 35(2):185–93. https://doi.org/10.1200/JCO.2016.67.1875 PMID: 28656203

59. Krönke J, Schlenk RF, Jensen KO, Tschürtz F, Corbacioglu A, Gaidzik VI, et al. Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2011; 29(19):2709–16. https://doi.org/10.1200/JCO.2011.35.0371 PMID: 21555683

60. Shayegi N, Kramer M, Bornhäuser M, Schaich M, Schetelig J, Platzbecker U, et al. The level of residual disease based on mutant NPM1 is an independent prognostic factor for relapse and survival in AML. Blood. 2013; 122(1):83–92. https://doi.org/10.1182/blood-2012-10-461749 PMID: 23656730

61. Stahl T, Badbaran A, Kröger N, Klyuchnikov E, Zabelina T, Zeschke S, et al. Minimal residual disease diagnostics in patients with acute myeloid leukemia in the post-transplant period: comparison of peripheral blood and bone marrow analysis. Leukemia & lymphoma. 2010; 51(10):1837–43. https://doi.org/10.3109/10428194.2010.508822 PMID: 20849383

62. Hubmann M, Köhnke T, Hoster E, Schneider S, Dufour A, Zellmeier E, et al. Molecular response assessment by quantitative real-time polymerase chain reaction after induction therapy in NPM1-mutated patients identifies those at high risk of relapse. Haematologica. 2014; 99(8):1317–25. https://doi.org/10.3324/haematol.2014.104133 PMID: 24816240
myeloid leukemias. Blood. 2010; 115(2):198–205. https://doi.org/10.1182/blood-2009-04-212530 PMID: 19901261

64. Eisfeld AK, Kohlschmidt J, Mims A, Nicolet D, Walker CJ, Blachly JS, et al. Additional gene mutations may refine the 2017 European LeukemiaNet classification in adult patients with de novo acute myeloid leukemia aged <60 years. Leukemia. 2020; 34(12):3215–27. https://doi.org/10.1038/s41375-020-0872-3 PMID: 32461631

65. Schlenk RF, Frech P, Weber D, Brossart P, Horst HA, Kraemer D, et al. Impact of pretreatment characteristics and salvage strategy on outcome in patients with relapsed acute myeloid leukemia. Leukemia. 2017; 31(5):1217–20. https://doi.org/10.1038/leu.2017.22 PMID: 28096533

66. Lussana F, Caprioli C, Stefanoni P, Pavoni C, Spinelli O, Buklijas K, et al. Molecular Detection of Minimal Residual Disease before Allogeneic Stem Cell Transplantation Predicts a High Incidence of Early Relapse in Adult Patients with NPM1 Positive Acute Myeloid Leukemia. Cancers (Basel). 2019; 11(10). https://doi.org/10.3390/cancers11101455 PMID: 31569375

67. Bataller A, Oñate G, Díaz-Beyá M, Guijarro F, Garrido A, Vives S, et al. Acute myeloid leukemia with NPM1 mutation and favorable European LeukemiaNet category: outcome after preemptive intervention based on measurable residual disease. Br J Haematol. 2020; 191(1):52–61. https://doi.org/10.1111/bjh.16857 PMID: 32510599

68. Platzbecker U, Middeke JM, Sockel K, Herbst R, Wolf D, Baldus CD, et al. Measurable residual disease-guided treatment with azacitidine to prevent haematological relapse in patients with myelodysplastic syndrome and acute myeloid leukaemia (RELAZ2): an open-label, multicentre, phase 2 trial. The Lancet Oncology. 2018; 19(12):1668–73. https://doi.org/10.1016/S1470-2045(18)30590-1 PMID: 30442503

69. Bill M, Grimm J, Jentsch M, Kloos L, Goldmann K, Schulz J, et al. Digital droplet PCR-based absolute quantification of pre-transplant NPM1 mutation burden predicts relapse in acute myeloid leukemia patients. Ann Hematol. 2018; 97(10):1757–65. https://doi.org/10.1007/s00277-018-3373-y PMID: 29785446

70. Karas M, Steinerova K, Lysak D, Hrabeťová M, Jungova A, Sramek J, et al. Pre-transplant Quantitative Determination of NPM1 Mutation Significantly Predicts Outcome of Allogeneic Hematopoietic Stem Cell Transplantation in Patients with Normal Karyotype AML in Complete Remission. Anticancer Res. 2016; 36(10):5487–92. https://doi.org/10.21873/anticancerres.11130 PMID: 27798920

71. Kayser S, Bennet A, Thiede C, Martens U, Huber J, Stadtherr P, et al. Pretransplant NPM1 MRD levels predict outcome after allogeneic hematopoietic stem cell transplantation in patients with acute myeloid leukemia. Blood Cancer J. 2016; 6(7):e449. https://doi.org/10.1038/bcj.2016.46 PMID: 27471865

72. Hourigan CS, Dillon LW, Gui G, Logan BR, Fei M, Ghannam J, et al. Impact of Conditioning Intensity of Allogeneic Transplantation for Acute Myeloid Leukemia With Genomic Evidence of Residual Disease. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2020; 38 (12):1273–83. https://doi.org/10.1200/JCO.19.03011 PMID: 31860405

73. Burchert A, Bug G, Fritz LV, Finke J, Stelljes M, Röllig C, et al. Sorafenib Maintenance After Allogeneic Hematopoietic Stem Cell Transplantation for Acute Myeloid Leukemia With FLT3-Internal Tandem Duplication Mutation (SORMAIN). Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2020; 38(26):2993–3002.

74. Kico JM, Miller CA, Griffith M, Petti A, Spencer DH, Kettar-Kulkarni S, et al. Association Between Mutaion Clearance After Induction Therapy and Outcomes in Acute Myeloid Leukemia. Jama. 2015; 314 (8):811–22. https://doi.org/10.1001/jama.2015.9643 PMID: 26305651

75. Lindsley RC, Mar BG, Mazzola E, Grauman PV, Shareef S, Allen SL, et al. Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. Blood. 2015; 125(9):1367–76. https://doi.org/10.1182/blood-2014-11-610543 PMID: 25550361

76. Stein EM, DiNardo CD, Fathi AT, Mims AS, Pratz KW, Savona MR, et al. Ivosidenib or enasidenib combined with intensive chemotherapy in patients with newly diagnosed AML: a phase 1 study. Blood. 2020.