Assessment of Minimal Residual Disease in Standard-Risk AML

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DOI: 10.1056/NEJMoa1507471

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Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):

Link to publication on Research at Birmingham portal

Publisher Rights Statement:

Validated Feb 2016

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Assessment of Minimal Residual Disease in Standard-Risk AML


BACKGROUND

Despite the molecular heterogeneity of standard-risk acute myeloid leukemia (AML), treatment decisions are based on a limited number of molecular genetic markers and morphology-based assessment of remission. Sensitive detection of a leukemia-specific marker (e.g., a mutation in the gene encoding nucleophosmin [NPM1]) could improve prognostication by identifying submicroscopic disease during remission.

METHODS

We used a reverse-transcriptase quantitative polymerase-chain-reaction assay to detect minimal residual disease in 2569 samples obtained from 346 patients with NPM1-mutated AML who had undergone intensive treatment in the National Cancer Research Institute AML17 trial. We used a custom 51-gene panel to perform targeted sequencing of 223 samples obtained at the time of diagnosis and 49 samples obtained at the time of relapse. Mutations associated with preleukemic clones were tracked by means of digital polymerase chain reaction.

RESULTS

Molecular profiling highlighted the complexity of NPM1-mutated AML, with segregation of patients into more than 150 subgroups, thus precluding reliable outcome prediction. The determination of minimal-residual-disease status was more informative. Persistence of NPM1-mutated transcripts in blood was present in 15% of the patients after the second chemotherapy cycle and was associated with a greater risk of relapse after 3 years of follow-up than was an absence of such transcripts (82% vs. 30%; hazard ratio, 4.80; 95% confidence interval [CI], 2.95 to 7.80; P<0.001) and a lower rate of survival (24% vs. 75%; hazard ratio for death, 4.38; 95% CI, 2.57 to 7.47; P<0.001). The presence of minimal residual disease was the only independent prognostic factor for death in multivariate analysis (hazard ratio, 4.84; 95% CI, 2.57 to 9.15; P<0.001). These results were validated in an independent cohort. On sequential monitoring of minimal residual disease, relapse was reliably predicted by a rising level of NPM1-mutated transcripts. Although mutations associated with preleukemic clones remained detectable during ongoing remission after chemotherapy, NPM1 mutations were detected in 69 of 70 patients at the time of relapse and provided a better marker of disease status.

CONCLUSIONS

The presence of minimal residual disease, as determined by quantitation of NPM1-mutated transcripts, provided powerful prognostic information independent of other risk factors. (Funded by Bloodwise and the National Institute for Health Research; Current Controlled Trials number, ISRCTN55675535.)
Although acute myeloid leukemia (AML) is genetically less complex than many other tumors, the condition is molecularly heterogeneous. Despite improved understanding of the mutational landscape, treatment decisions, particularly regarding allogeneic stem-cell transplantation, remain guided by cytogenetic analysis, a limited panel of molecular genetic markers, and morphology-based assessment of remission.

Currently, a predicted risk of relapse of more than 35% is widely considered to warrant stem-cell transplantation during the first remission. Patients with high-risk disease undergo stem-cell transplantation if feasible, whereas those with low-risk disease usually do not. However, there is uncertainty about the role of transplantation in patients with cytogenetically standard-risk AML (most of whom have cytogenetically normal AML), which affects approximately 50% of younger adult patients. The most common molecular lesion in this group (which is present in approximately a third of patients with AML and more than half of those with cytogenetically normal AML) is a mutation in the gene encoding nucleophosmin (NPM1). The outcome of disease treatment is influenced by the presence or absence of cooperating internal tandem duplications in the gene encoding Fms-like tyrosine kinase 3 (FLT3-ITD) and of mutations in the gene encoding DNA methyltransferase 3A (DNMT3A).

Patients who have mutated NPM1 without the FLT3-ITD genotype have a comparatively better outcome than those with coexisting FLT3-ITD mutations and hence are no longer recommended for transplantation for the first remission. In contrast, patients with mutated NPM1 and coexisting FLT3-ITD or DNMT3A mutations (who account for approximately 66% of patients with NPM1-mutated AML) have a poorer prognosis and may be considered as candidates for transplantation.

There is growing interest in whether prognostication can be improved through more extensive molecular profiling that capitalizes on advances in sequencing technology. In addition, the risk of relapse may be better defined with the use of leukemia-specific molecular markers (e.g., mutated NPM1) as targets for detection of sub-microscopic levels of leukemia, so-called minimal residual disease, after therapy. Although the presence of minimal residual disease can provide prognostic information, it is unclear whether such identification is useful in the context of systematic molecular profiling. Moreover, questions have been raised about the assessment of minimal residual disease in routine practice, given the clonal complexity of AML. Recent studies have indicated that markers such as mutated NPM1 may not always be stable over the course of disease and that relapse potentially emanates from preleukemic clones. To address these issues, we undertook molecular profiling and sequential monitoring of minimal residual disease in a large series of patients with NPM1-mutated AML who had undergone intensive treatment in the National Cancer Research Institute (NCRI) AML17 trial.

**METHODS**

**PATIENTS**

Patients were enrolled in the NCRI AML17 trial from April 6, 2009, to December 31, 2014. (A list of treatments is provided in Fig. S1 in Supplementary Appendix 1, available with the full text of this article at NEJM.org.) Post-remission treatment (consolidation therapy) was determined according to the Medical Research Council (MRC) risk score, with poor-risk patients recommended for stem-cell transplantation during the first remission. (The MRC risk score can range from 1.28 to 4.76 or more, with higher values indicating greater risk.) Centralized molecular screening identified patients with NPM1 mutations. Follow-up samples were scheduled to be obtained at the time of blood-count regeneration after each cycle of treatment and then quarterly until 24 months after consolidation therapy. Samples that were obtained at early time points (i.e., on regeneration after induction and consolidation cycles) were mostly samples of peripheral blood, since the evaluation of bone marrow was prioritized for flow cytometry.

In the development phase of this study (from April 2009 through May 2012), clinicians were not informed about the status of minimal residual disease so that its prognostic value could be reliably assessed. In June 2012, we observed that molecular relapse was associated with disease progression. From that time on, we prospectively analyzed follow-up samples and immediately informed clinicians of the results. Patients who were recruited between June 2012 and December 2014 made up a validation cohort.
Amplification of NPM1-Mutated Transcripts

To amplify NPM1-mutated transcripts, we prepared complementary DNA from total RNA, as described previously.29 We detected minimal residual disease on reverse-transcriptase quantitative polymerase-chain-reaction (RT-qPCR)16 using a mutation-specific primer with a common primer and probe (Fig. 1, and Table S1 in Supplementary Appendix 1). To detect rare mutations, we designed patient-specific primers (Primer Express, Applied Biosystems). Assays were run in triplicate on the ABI 7900 platform (Applied Biosystems), and mutated transcript levels were compared to expression of the ABL1 reference gene with the use of plasmid standards (Qiagen), with the difference-in-cycle-thresholds method used for rare mutations.29 Assays were performed under PCR conditions proposed by the Europe Against Cancer (EAC) program30 except for the annealing temperature, which was adjusted to eliminate background amplification (Table S1 in Supplementary Appendix 1). Follow-up samples with
ABL1 cycle-threshold values of 30 or more were excluded from the analysis. RT-qPCR positivity for mutated NPM1 was defined according to amplification in at least two of three replicates with cycle-threshold values of 40 or less (using a threshold setting of 0.1), according to EAC criteria.30

WHOLE-EXOME SEQUENCING, MOLECULAR PROFILING, AND DIGITAL PCR
Genomic DNA libraries that had been prepared with the use of the SureSelectXT Human All Exon V4 target-enrichment system (Agilent Technologies) were sequenced on the Illumina HiSeq 2000 platform with 100-bp paired-end reads. (Details regarding read alignment and variant calling are provided in the Methods section in Supplementary Appendix 1.)

We developed a custom-targeted sequencing panel of 51 genes31 that was based on published data for NPM1-mutated AML2 and on exome sequencing of samples obtained from 22 patients in the study cohort (Table S2 in Supplementary Appendix 1 and Table S3 in Supplementary Appendix 2). High-throughput sequencing with the use of HaloPlex Target Enrichment (Agilent Technologies) was performed on the Illumina HiSeq 2000.31 Diagnostic DNA from 223 samples obtained from patients with NPM1-mutated AML was sequenced, and additional mutations (median number, 3; range, 1 to 8) were identified in 222 samples (99.6%), with a median read depth of 1280× (range, 51 to 6700). We determined the frequencies of mutated NPM1 and FLT3-ITD alleles in parallel using GeneScan analysis.32 (Details regarding digital PCR assays are provided in the Methods section in Supplementary Appendix 1.)

STUDY END POINTS
All end points were based on the revised criteria of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia, except that the recovery of cell counts in peripheral blood was not required for a determination of complete remission.33 The definition of molecular remission was an absence of detectable NPM1-mutated transcripts on RT-qPCR in a bone marrow sample affording a sensitivity of at least 1 in 10,000. Among patients who had a molecular remission, a molecular relapse was defined as the detection of increasing levels of NPM1-mutated transcripts in two successive samples in the absence of hematologic relapse. Median follow-up for survival was 40.5 months in the development cohort and 13.6 months in the validation cohort.

STATISTICAL ANALYSIS
We used Kaplan–Meier estimates or the competing-risks method to calculate survival percentages. Time-to-event analyses were performed with either the log-rank test or Cox regression, with the effect of transplantation analyzed by means of the Mantel–Byar method. We used Cox regression with forward selection to identify independent prognostic factors. All reported P values are two-sided, and P values of less than 0.05 were considered to indicate statistical significance.

RESULTS
DETECTION OF MINIMAL RESIDUAL DISEASE IN PATIENTS WITH NPM1 MUTATIONS
The preliminary development phase involved 346 patients with NPM1 mutations (median age, 50 years; range, 6 to 68) who had available follow-up samples. We used mutation-specific reverse primers on RT-qPCR to detect the presence of minimal residual disease in patients who carried a total of 27 different mutations (Fig. 1, and Table S1 in Supplementary Appendix 1). The median sensitivity, as determined with the use of diagnostic samples, was 1.0×10−3 (range, 1.0×10−3.7 to 1.0×10−7.1).

EARLY ASSESSMENT OF MINIMAL RESIDUAL DISEASE AND RELAPSE RISK
In the development phase, we analyzed 2569 follow-up samples that could be evaluated (902 bone marrow samples and 1667 peripheral-blood samples), for a median of 6 samples per patient. During therapy, we observed higher rates of detection of minimal residual disease in bone marrow than in peripheral blood. Nevertheless, the presence of minimal residual disease in peripheral blood was highly informative among patients in complete remission on morphologic analysis. The persistence of NPM1-mutated transcripts in peripheral blood after regeneration following the second chemotherapy cycle was associated with a significantly higher risk of relapse at 3 years than was the absence of such transcripts (82% vs. 30%; univariate hazard ratio, 4.80; 95% confidence interval [CI], 2.95 to 7.80; P<0.001) and a lower rate of survival (24% vs. 75%; univariate
The hazard ratio for death, 4.38; 95% CI, 2.57 to 7.47; P<0.001) (Table 1 and Fig. 2). The presence of minimal residual disease in peripheral blood among patients in complete remission on morphologic analysis after the second chemotherapy cycle was more discriminatory than RT-qPCR positivity in peripheral blood at any other time point or in bone marrow at any time point during therapy (Table S4 in Supplementary Appendix 1). After adjustment for minimal-residual-disease status in peripheral blood following the second chemotherapy cycle, no other measurement of minimal residual disease provided additional prognostic value.

**Minimal-Residual-Disease Status and Mutation Profile**

To assess whether the status of minimal residual disease in peripheral blood after the second chemotherapy cycle was an independent prognostic factor, we performed univariate and multivariate analyses (Table 1) and included the results of sequencing analyses (which identified 161 unique genetic subgroups or 76 subgroups if only the 10 most common genetic targets were considered) (Fig. 3). On univariate analysis, the risk of relapse was significantly higher among patients with an increased white-cell count, the presence

<table>
<thead>
<tr>
<th>Mutated Gene or Category</th>
<th>Patients</th>
<th>Risk of Relapse</th>
<th>Risk of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no./total no.</td>
<td>Hazard Ratio (95% CI)</td>
<td>P Value</td>
</tr>
<tr>
<td><strong>DNMT3A</strong></td>
<td>73/149</td>
<td>1.95 (1.17–3.23)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>FLT3-ITD</strong></td>
<td>74/190</td>
<td>1.63 (1.03–2.57)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>FLT3-TKD</strong></td>
<td>27/190</td>
<td>1.30 (0.70–2.41)</td>
<td>0.41</td>
</tr>
<tr>
<td><strong>NRAS</strong></td>
<td>24/149</td>
<td>0.69 (0.33–1.46)</td>
<td>0.34</td>
</tr>
<tr>
<td>Cohesin†</td>
<td>28/149</td>
<td>0.72 (0.36–1.46)</td>
<td>0.36</td>
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<tr>
<td><strong>PTPN11</strong></td>
<td>24/149</td>
<td>0.98 (0.48–1.98)</td>
<td>0.95</td>
</tr>
<tr>
<td><strong>TET2</strong></td>
<td>24/149</td>
<td>1.29 (0.69–2.42)</td>
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</tr>
<tr>
<td><strong>IDH1</strong></td>
<td>11/149</td>
<td>0.73 (0.27–2.02)</td>
<td>0.55</td>
</tr>
<tr>
<td><strong>IDH2</strong></td>
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<td>1.12 (0.60–2.10)</td>
<td>0.73</td>
</tr>
<tr>
<td><strong>WT1</strong></td>
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<td>1.56 (0.77–3.17)</td>
<td>0.21</td>
</tr>
<tr>
<td>Age per yr</td>
<td>NA</td>
<td>1.00 (0.98–1.02)</td>
<td>0.89</td>
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<tr>
<td>White-cell count per log increase</td>
<td>NA</td>
<td>1.90 (1.24–2.91)</td>
<td>0.003</td>
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<tr>
<td>Abnormal result on cytogenetic analysis</td>
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<td>1.59 (0.69–3.69)</td>
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<tr>
<td>Increase in MRC risk group</td>
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<td>1.16 (0.76–1.76)</td>
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</tr>
<tr>
<td>MRD-positive status</td>
<td>30/194</td>
<td>4.80 (2.95–7.80)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Listed are the results of univariate analysis of data from patients for whom the status of minimal residual disease (MRD) was determined in peripheral-blood samples obtained after the second cycle of chemotherapy. In multivariate analyses, only MRD-positive status was significant.

† Cohesin complex includes RAD21, SMC1A, SMC3, and STAG2.

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**Table 1. Risk of Relapse or Death in Study Cohort on Univariate Analysis.**

**Figure 2 (facing page). Minimal Residual Disease in Peripheral Blood after the Second Cycle of Chemotherapy and Clinical Outcomes.**

Shown are the rates of overall survival (Panel A) and the cumulative incidence of relapse in all patients (Panel B), in those without FLT3-ITD mutations (Panel C) and those with FLT3-ITD mutations (Panel D), in those without DNMT3A mutations (Panel E) and those with DNMT3A mutations (Panel F) among patients who were found to have minimal residual disease (MRD-positive) or no minimal residual disease (MRD-negative) in peripheral-blood samples. Censoring of data is indicated by black tick marks, and death during remission is indicated by green tick marks.
Minimal Residual Disease in Standard-Risk AML

A Overall Survival

<table>
<thead>
<tr>
<th></th>
<th>No. of Patients</th>
<th>No. of Events</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>MRD-negative</td>
<td>164</td>
<td>40</td>
<td>&lt;0.001</td>
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<tr>
<td>MRD-positive</td>
<td>30</td>
<td>21</td>
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</table>

![Graph A](image)

B Relapse in All Patients

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<th>No. of Patients</th>
<th>No. of Events</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>MRD-negative</td>
<td>164</td>
<td>50</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MRD-positive</td>
<td>30</td>
<td>25</td>
<td></td>
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</tbody>
</table>

![Graph B](image)

C Relapse in Patients without FLT3-ITD Mutations

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<th>No. of Patients</th>
<th>No. of Events</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRD-negative</td>
<td>105</td>
<td>30</td>
<td>0.001</td>
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<tr>
<td>MRD-positive</td>
<td>11</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

![Graph C](image)

D Relapse in Patients with FLT3-ITD Mutations

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<th>No. of Patients</th>
<th>No. of Events</th>
<th>P-value</th>
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<tr>
<td>MRD-negative</td>
<td>56</td>
<td>19</td>
<td>0.001</td>
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<tr>
<td>MRD-positive</td>
<td>18</td>
<td>16</td>
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</table>

![Graph D](image)

E Relapse in Patients without DNMT3A Mutations

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<th>No. of Events</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>MRD-negative</td>
<td>70</td>
<td>19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MRD-positive</td>
<td>6</td>
<td>6</td>
<td></td>
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</table>

![Graph E](image)

F Relapse in Patients with DNMT3A Mutations

<table>
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<th>No. of Patients</th>
<th>No. of Events</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRD-negative</td>
<td>56</td>
<td>25</td>
<td>0.004</td>
</tr>
<tr>
<td>MRD-positive</td>
<td>17</td>
<td>13</td>
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</table>

![Graph F](image)
Figure 3. Mutation Status of Genes in 223 Samples of NPM1-Mutated AML.

Targeted gene sequencing was performed with a custom 51-gene panel. The genes represented on this panel were selected on the basis of the Cancer Genome Atlas (TCGA) data and on data obtained by exome-sequencing analysis of 19 diagnostic samples and 11 relapse samples obtained from 22 study cohort patients; of these patients, 5 were in ongoing complete remission and 17 were subject to relapse. The genes represented on this panel were CCND3, CTCF, KDM3B, KDM6A, KMT2D, LZTR1, MYC, NIPBL, POT1, SS18, and ZBTB7A. (Additional details are provided in Table S3 in Supplementary Appendix 2.) Each column represents an individual patient, with mutations grouped into functional categories. Mutated status is indicated in black, and wild-type status in white. Shown at the bottom of the graph are data for patients who were separated into subgroups on the basis of sharing the same combination of mutations among the top 10 mutational targets — NPM1, DNMT3A, IDH1, IDH2, TET2, WT1, FLT3-ITD, FLT3 point mutation (FLT3-PM), NRAS, and PTPN11 — and among mutations in the full panel of genes. Also shown is the status of patients with respect to minimal residual disease (MRD) in peripheral blood after two cycles of chemotherapy on RT-qPCR assay; black indicates MRD-positive, white indicates MRD-negative, and gray indicates that no sample was available.
of DNMT3A and FLT3-ITD mutations, and the presence of minimal residual disease in peripheral blood after the second chemotherapy cycle. Only an increased white-cell count and the presence of minimal residual disease were significantly associated with the rate of survival. We could find no specific molecular subgroup consisting of 10 patients or more that had a rate of survival of less than 52%; in contrast, the rate in the group with the presence of minimal residual disease was 24%. Patients with minimal residual disease in peripheral blood after the second chemotherapy cycle were more likely to have a high MRC risk score (i.e., >2.6667) than were those without minimal residual disease (50% vs. 16%, P<0.001) and to carry the FLT3-ITD mutation (62% vs. 35%, P=0.006). However, among patients who had AML with mutated FLT3-ITD, there was no significant difference in the allelic burden between those with minimal residual disease and those without minimal residual disease.

In multivariate modeling with forward selection, the presence of minimal residual disease was the only significant prognostic factor for relapse (hazard ratio, 5.09; 95% CI, 2.84 to 9.13; P<0.001) or death (hazard ratio, 4.84; 2.57 to 9.15; P<0.001). Among patients without one of the mutations associated with increased risk (FLT3-ITD and DNMT3A), those with minimal residual disease had a poorer outcome than did those without minimal residual disease (Fig. 2). Conversely, among patients with a high-risk genotype (FLT3-ITD, mutated DNMT3A, or both), negative results on RT-qPCR assay of peripheral blood after the second chemotherapy cycle distinguished 93 of 117 patients (79%) who had a relatively favorable outcome (rate of survival, 76%). After adjustment for the status of minimal residual disease, additional prognostic information about survival was not provided by the presence of mutations in FLT3-ITD (P=0.45) or in DNMT3A (P=0.93) or by the MRC risk group (P=0.98).

**MINIMAL RESIDUAL DISEASE AND PROGNOSIS IN THE VALIDATION COHORT**

To ensure that our findings were replicable, we investigated the status of minimal residual disease in a validation cohort of 91 patients with NPM1 mutations who were enrolled in the AML17 study starting in June 2012. The analyses confirmed that the presence of minimal residual disease in peripheral blood on RT-qPCR assay after the second chemotherapy cycle predicted a worse outcome at 2 years than did the absence of minimal residual disease, with an increased cumulative incidence of relapse (70% vs. 31%, P=0.001) and a lower rate of overall survival (40% vs. 87%, P=0.001) (Fig. 4).

In sensitivity analyses of data from the entire cohort to identify any effect of additional treatments, there were no significant interactions with any randomized therapy. However, there was a potential interaction with the use of gemtuzumab ozogamicin (P=0.07 for interaction), with less effect of minimal residual disease among patients receiving the drug, although the numbers were small (5 patients without minimal residual disease vs. 8 patients with minimal residual disease); at 5 years, the rates of survival among patients who received gemtuzumab ozogamicin were 63% among patients without minimal residual disease and 33% among those with minimal residual disease.

In the entire cohort, stem-cell transplantation was performed in 21 of 46 patients (46%) with minimal residual disease and in 61 of 239 patients (26%) without minimal residual disease (P=0.006). Among patients with FLT3-ITD mutations, stem-cell transplantation was performed in 12 of 27 patients (44%) with minimal residual disease and in 22 of 81 patients (27%) without minimal residual disease; among patients without FLT3-ITD mutations, stem-cell transplantation was performed in 9 of 18 patients (50%) with minimal residual disease and in 38 of 155 patients (25%) without minimal residual disease. Among patients with DNMT3A mutations, stem-cell transplantation was performed in 5 of 17 patients (29%) with minimal residual disease and in 20 of 56 patients (36%) without minimal residual disease; among patients without DNMT3A mutations, stem-cell transplantation was performed in 3 of 6 patients (50%) with minimal residual disease and in 15 of 70 patients (21%) without minimal residual disease.

In a sensitivity analysis in which survival was censored at the time of stem-cell transplantation, the absence of minimal residual disease remained prognostic (hazard ratio, 0.11; 95% CI, 0.05 to 0.26; P<0.001). On Mantel–Byar analysis, there was no significant effect of transplantation overall or among patients according to status with respect to minimal residual disease, although the numbers of patients were small.
### Table: Subgroup Analysis of MRD-Positive and MRD-Negative Patients

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>MRD-Positive</th>
<th>MRD-Negative</th>
<th>Statistics</th>
<th>Hazard Ratio (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no. of events/ no. of patients</td>
<td>O−E</td>
<td>Variance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relapse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Development</td>
<td>25/30</td>
<td>50/164</td>
<td>17.7</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>Validation</td>
<td>9/16</td>
<td>13/75</td>
<td>5.5</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Subtotal</td>
<td>34/46</td>
<td>63/239</td>
<td>23.3</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>Test of heterogeneity between subgroups: χ² = 1.4; P=0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Death    |              |              |            |                      |         |
| Development | 21/30         | 40/164       | 14.4       | 5.9                  |         |
| Validation | 7/16          | 6/75         | 4.5        | 2.0                  |         |
| Subtotal  | 28/46         | 46/239       | 18.9       | 7.9                  |         |
| Test of heterogeneity between subgroups: χ² = 0.0; P=0.83 | | | | | |

**Figure 4. Minimal Residual Disease as a Predictor of Outcome in Development and Validation Cohorts.**

Shown are the results of stratified log-rank analysis of relapse and overall survival among patients who were found to have minimal residual disease (MRD-positive) as compared with those who were found to have no minimal residual disease (MRD-negative) in peripheral blood after the second cycle of chemotherapy in both the development and the validation cohorts. The black squares indicate odds ratios, with the size of the square proportional to the number of patients in the subgroup. The diamonds indicate the overall values for the development and validation cohorts combined. The test of heterogeneity was performed with the use of the chi-square test with one degree of freedom. Proportions calculated from numbers of patients shown here may not equal the 3-year Kaplan–Meier or cumulative incidence rates reported in the text because of variable lengths of follow-up and the occurrence of events beyond 3 years. O−E indicates the observed number of events minus the expected number of events.

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**Sequential RT-qPCR Monitoring in Prediction of Relapse Among Patients with NPM1 Mutations**

The results of sequential monitoring of samples that were obtained after the end of consolidation treatment were available for 243 patients with NPM1 mutations in the development cohort. Among 53 patients in morphologic remission, a rising NPM1-mutated transcript level in preceding samples on RT-qPCR reliably predicted the occurrence of a hematologic relapse (median increment, 0.7 log₁₀ per month; range, 0.3 to 2.0) (Fig. S2 in Supplementary Appendix 1). Serial monitoring of paired samples of bone marrow and peripheral blood showed that the analysis of bone marrow increased the rate of detection of minimal residual disease, affording a median 1-log₁₀ increment in sensitivity (Fig. S3 in Supplementary Appendix 1). The analysis of the time from the first molecular positivity to relapse showed a longer time to relapse if minimal residual disease was first detected in bone marrow than if it was first detected in peripheral blood (median, 133 days vs. 87 days; P=0.65). Molecular relapse was diagnosed in an additional 11 patients; of these patients, 10 received preemptive therapy and 1 died from a cardiac event while in clinical remission.

Targeted sequencing at the time of molecular or hematologic relapse showed that the variant allele frequencies in some genes exceeded those in NPM1 in 34 of 49 patients (69%) who were tested, a finding that was consistent with acquisition of uniparental disomy in the case of FLT3-ITD and the probable presence of preleukemic clones for DNMT3A, TET2, and IDH1 mutations (Fig. S4 in Supplementary Appendix 1). In addition, mutations in DNMT3A, TET2, and IDH2 were detected at low levels in remission samples that were used as controls for exome sequencing and that were negative for NPM1 mutations at a sensitivity of at least 1.0×10⁻⁴ (Table S3 in Supplementary Appendix 2). Regardless of mutations associated with preleukemic clones, RT-qPCR analysis of available relapse samples showed that NPM1 mutations were a stable marker of AML disease status and were detectable in 69 of 70 patients (99%) at the time of relapse. This finding argues against the theory that relapse commonly arises from wild-type NPM1 preleukemic clones.
mic clones. In the remaining patient, the diagnosis of relapse was based on the morphologic detection of 9% blasts in a sample of bone marrow after the third chemotherapy cycle. Targeted sequencing of DNA derived from some of these blasts with the use of the 51-gene panel showed no mutations, including the FLT3 D835Y mutation (in 1066 reads) and the PTPN11 F71L mutation (in 1261 reads) that were detected before treatment, which calls into question the diagnosis of relapse.

To investigate the most effective approach for tracking minimal residual disease, digital PCR was performed in follow-up samples obtained from 53 patients with NPM1-mutated AML who had diagnostic DNMT3A or IDH1/2 hotspot mutations and who were in the first complete remission on morphologic analysis and in molecular remission according to the NPM1-mutated RT-qPCR assay. Among 24 patients with DNMT3A R882H at diagnosis who received chemotherapy alone, all 24 who were tested at a median of 28 months (range, 2 to 40) showed high-level persistence of the mutation (at a median variant allele frequency of 21%; range, 1 to 49). Of these 24 patients, 16 (67%) remained in the first complete remission at a median follow-up of 38 months (range, 27 to 56). IDH1 R132H was detected at follow-up in 1 of 11 patients (9%) at a 1% level. IDH2 R140Q was detected in 8 of 18 patients (44%) who were tested at a median of 18 months (range, 8 to 45) at a median 2% level (range, 0.2 to 42), with 5 of the 8 patients (63%) remaining in the first complete remission at 36 months (range, 28 to 65). Allogeneic stem-cell transplantation led to the elimination of the DNMT3A mutant clone in 8 of 9 patients (89%) who were tested (Fig. S5 in Supplementary Appendix 1).

**Discussion**

Currently, treatment decisions for young adults with AML are based largely on cytogenetic analysis, other well-validated demographic data, and very few molecular genetic markers. However, such prognostication does not reflect other areas of heterogeneity. The assessment of minimal residual disease may capture some of this individual variation but to date is established for use only in acute promyelocytic leukemia and childhood acute lymphoblastic leukemia. We therefore investigated the prognostic value of assessment of minimal residual disease in the most common molecular subtype of AML.

Molecular profiling highlighted the molecular heterogeneity of AML, with 223 patients categorized into more than 150 subgroups. In terms of genetic changes that were identified, only the presence of FLT3-ITD and mutated DNMT3A had a significant effect on the outcome on univariate analysis. However, on multivariate analysis, the presence of minimal residual disease (i.e., the persistence of NPM1-mutated transcripts) in the peripheral blood of patients after the second chemotherapy cycle was highly discriminatory and the only significant prognostic factor. Among patients with a high-risk genotype (FLT3-ITD, mutated DNMT3A, or both), negative results on RT-qPCR assay of peripheral blood after the second chemotherapy cycle distinguished a group of patients (79%) with a relatively favorable outcome (rate of survival, 76%), which has implications for the value of stem-cell transplantation in this group. Even among patients with both FLT3-ITD and DNMT3A mutations, a negative result on RT-qPCR assay of peripheral blood after the second chemotherapy cycle was associated with a survival rate of 70% at 3 years. Conversely, slow clearance of minimal residual disease was used to define a subgroup of patients (10%) who had a favorable genotype (i.e., no FLT3-ITD and no mutation in DNMT3A) and yet a very poor outcome. Typically, these patients would not be considered for stem-cell transplantation during first complete remission. The prognostic value of minimal-residual-disease status in peripheral blood after the second chemotherapy cycle was confirmed in an independent cohort of patients who underwent prospective assessment of minimal residual disease.

Apart from refining risk stratification, assessment of leukemia-specific markers such as mutated NPM1 can be used for sequential monitoring of minimal residual disease to identify impending relapse. This strategy has been most widely applied in the treatment of patients with acute promyelocytic leukemia. However, some observers have questioned the suitability of the use of mutated NPM1 for monitoring of minimal residual disease, since this mutation can arise in the context of a preleukemic clone characterized by mutations in epigenetic landscaping genes (e.g., DNMT3A and IDH1/2) from which subsequent relapse may arise. Observation of variant allele frequencies in relapsing disease and results of digital PCR assays show-
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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank the clinicians, research nurses, and laboratory scientists who provided samples for molecular analyses from the NCRI AML17 trial centers (listed in Supplementary Appendix 1); Muddassar Mirza, Efterpi Papouli, and Alka Saxena of the BRC Genomics Laboratory, King's College London, for assistance in high-throughput mutational analyses; and David Bowen and Mike Dennis for their critical reading of an earlier version of the manuscript and helpful comments.

APPENDIX

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