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ORIGINAL ARTICLE

Assessment of Minimal Residual Disease in Standard-Risk AML

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ABSTRACT

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.)

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Although acute myeloid leukemia (AML) is genetically less complex than many other tumors, the condition is molecularly heterogeneous.[1-3] 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.[4-6]

Currently, a predicted risk of relapse of more than 35% is widely considered to warrant stem-cell transplantation during the first remission.[5] 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*).[7] 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*).[8-12] Patients who have mutated *NPM1* without the *FLT3*-ITD genotype have a comparatively better outcome than do those with coexisting *FLT3*-ITD mutations and hence are no longer recommended for transplantation during the first remission.[4-6,8] 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.[3,13,14] 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 submicroscopic levels of leukemia, so-called minimal residual disease, after therapy.[15] Although the presence of minimal residual disease can provide prognostic information,[16-22] 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.[23,24] 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.)[25] Post-remission treatment (consolidation therapy) was determined according to the Medical Research Council (MRC) risk score,[26,27] 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.[11,28] 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.

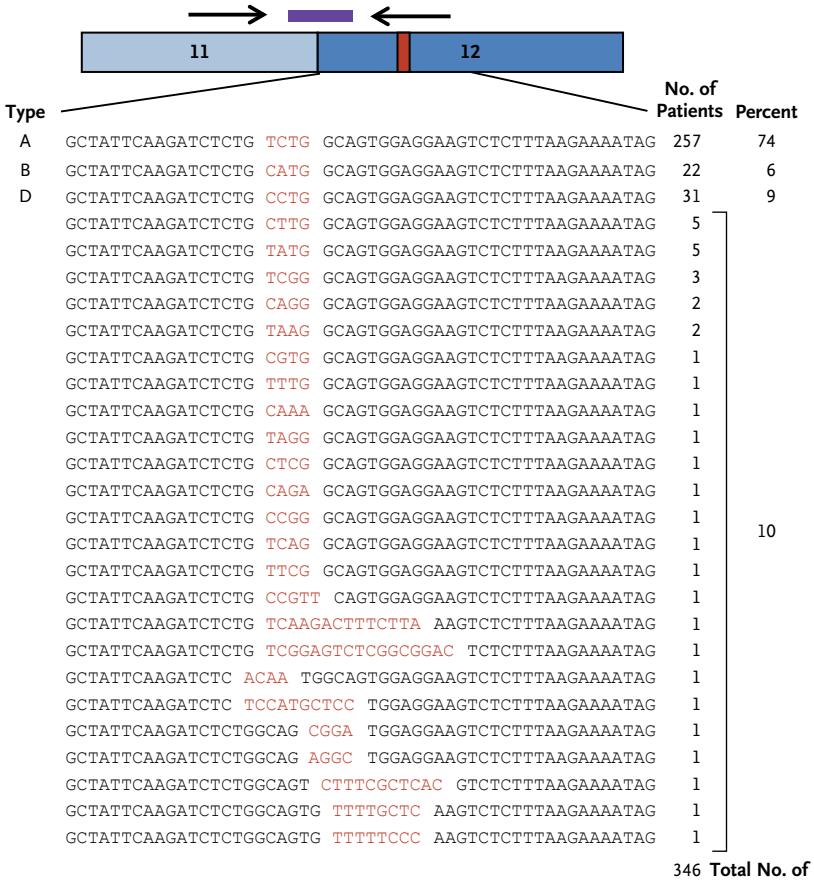


Figure 1. Detection of Minimal Residual Disease by Analysis of *NPM1*-Mutated Transcripts.

NPM1 mutations that were identified in 346 patients in the AML17 trial served as targets to track minimal residual disease by means of reverse-transcriptase quantitative polymerase-chain-reaction (RT-qPCR) assays with primers designed to amplify different mutant *NPM1* transcripts. Mutations that were identified on central molecular screening[11,28] were confirmed on PCR amplification and Sanger sequencing. All *NPM1* mutations (red) occurred in exon 12 and resulted in a net insertion of four base pairs. The three most common mutations (types A, B, and D) accounted for 90% of cases. Rare or unique mutations were found in the remaining 10%. On the basis of the assay described by Gorello and colleagues,[16] a common forward primer and probe (purple) were combined with a mutant-specific reverse primer to detect minimal residual disease. Additional details are provided in Table S1 in Supplementary Appendix 1.

The AML17 trial was approved by Wales Research Ethics Committee 3. All patients provided written informed consent.

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) program[30] 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 SureSelect^{XT} 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 genes[31] that was based on published data for *NPM1*-mutated AML[2] 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^{-5} (range, $1.0\times10^{-3.7}$ to $1.0\times10^{-7.1}$).

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

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

Mutated Gene or Category	Patients	Risk of Relapse		Risk of Death	
		Hazard Ratio (95% CI)	P Value	Hazard Ratio (95% CI)	P Value
	no./total no.				
DNMT3A	73/149	1.95 (1.17–3.23)	0.01	1.31 (0.74–2.31)	0.35
FLT3-ITD	74/190	1.63 (1.03–2.57)	0.04	1.56 (0.94–2.64)	0.09
FLT3-TKD	27/190	1.30 (0.70–2.41)	0.41	1.11 (0.52–2.33)	0.79
NRAS	24/149	0.69 (0.33–1.46)	0.34	0.82 (0.37–1.83)	0.62
Cohesin†	28/149	0.72 (0.36–1.46)	0.36	0.77 (0.34–1.71)	0.51
PTPN11	24/149	0.98 (0.48–1.98)	0.95	0.93 (0.42–2.07)	0.86
TET2	24/149	1.29 (0.69–2.42)	0.43	1.40 (0.70–2.82)	0.34
IDH1	11/149	0.73 (0.27–2.02)	0.55	0.77 (0.24–2.47)	0.66
IDH2	25/149	1.12 (0.60–2.10)	0.73	0.99 (0.46–2.12)	0.98
WT1	16/149	1.56 (0.77–3.17)	0.21	1.82 (0.85–3.88)	0.12
Age per yr	NA	1.00 (0.98–1.02)	0.89	1.02 (0.99–1.04)	0.20
White-cell count per log increase	NA	1.90 (1.24–2.91)	0.003	1.59 (1.00–2.53)	0.05
Abnormal result on cytogenetic analysis	21/178	1.59 (0.69–3.69)	0.28	1.27 (0.54–2.95)	0.58
Increase in MRC risk group	NA	1.16 (0.76–1.76)	0.50	1.26 (0.79–2.02)	0.34
MRD-positive status	30/194	4.80 (2.95–7.80)	<0.001	4.38 (2.57–7.47)	<0.001

* 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.

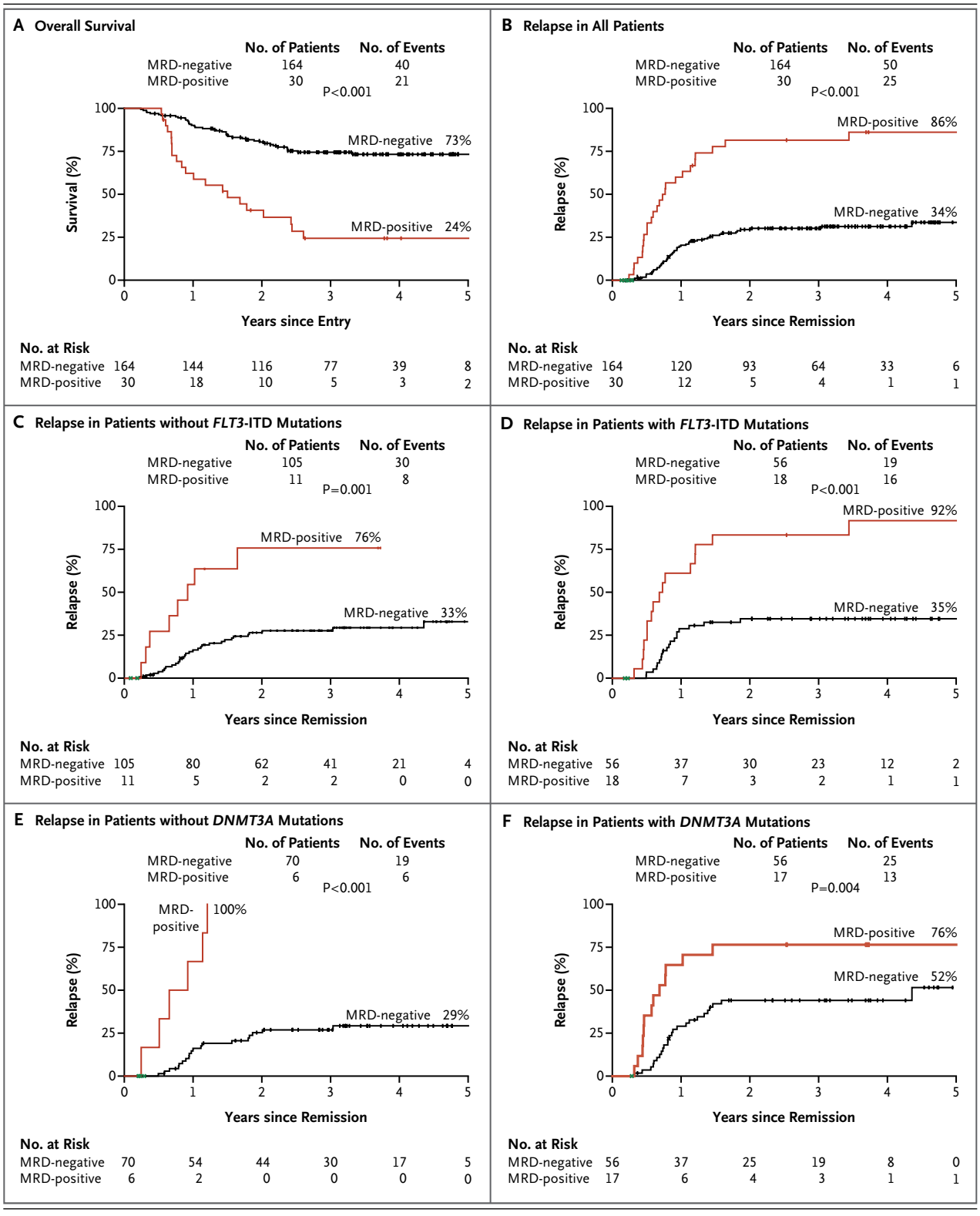
hazard ratio for death, 4.38; 95% CI, 2.57 to 7.47; P<0.001) (Table 1 and Fig. 2). The presence of minimal residual disease in peripheral blood among patients in complete remission on morphologic analysis after the second chemotherapy cycle was more discriminatory than RT-qPCR positivity in peripheral 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

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), and 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.



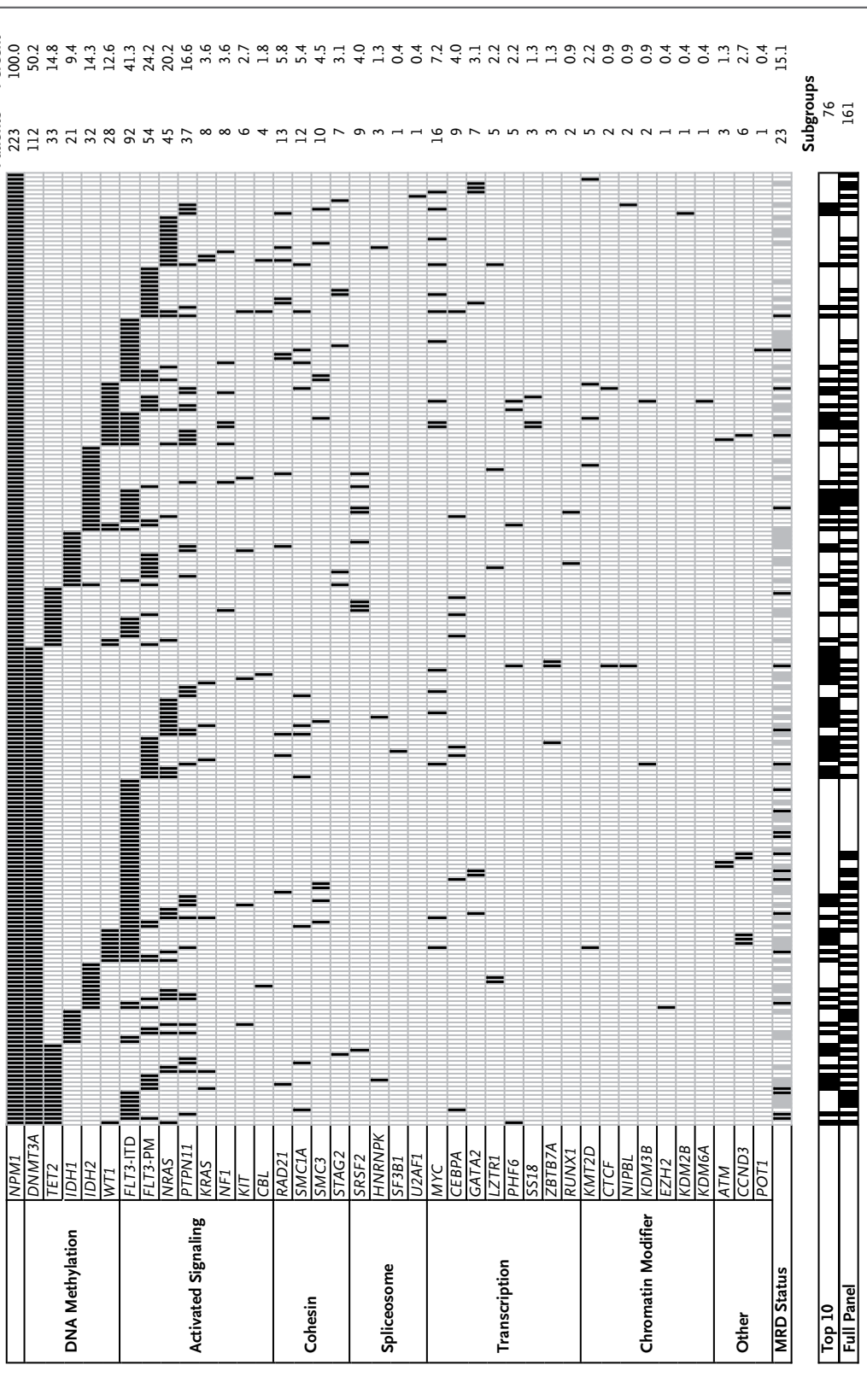


Figure 3. Mutation Status of Genes in 223 Samples of *NPM1*-Mutated AML.

Targeted gene sequencing was performed with a custom 51-gene panel.[31] The genes represented on this panel were selected on the basis of the Cancer Genome Atlas (TCGA) data[2] 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. On the basis of the exome-sequencing data, included in the 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.

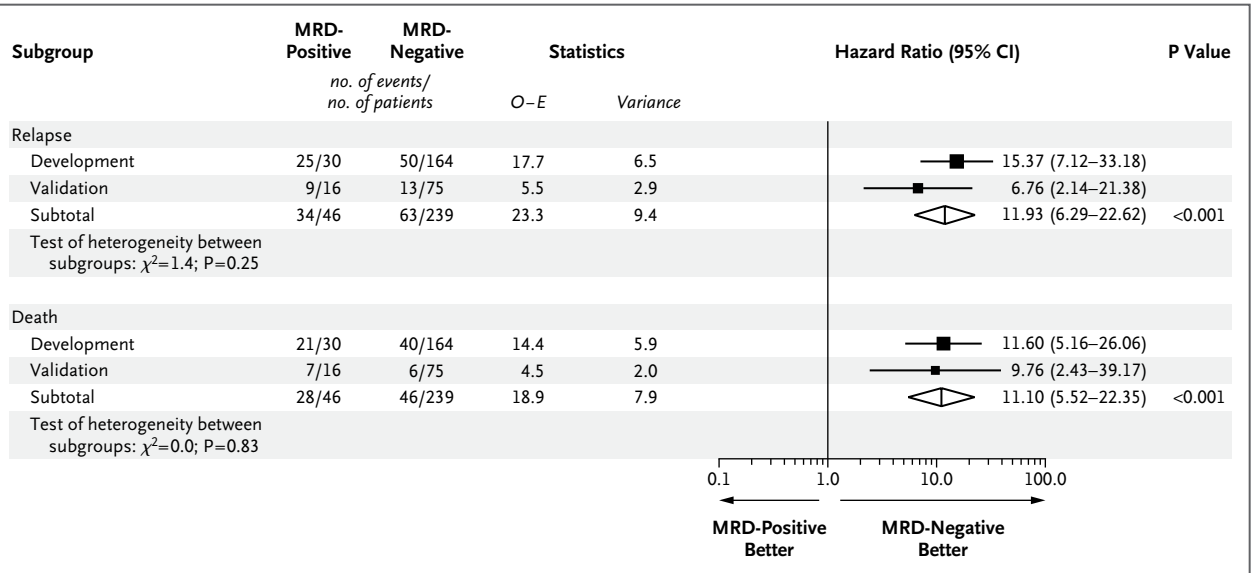


Figure 4. Minimal Residual Disease as a Predictor of Outcome in Development and Validation Cohorts.

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 events 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.

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 $\log_{10}$ 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-$\log_{10}$ 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[34] 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* preleuke-

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.[3-6,35,36] 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.[15,37,38] We therefore investigated the prognostic value of assessment of minimal residual disease in the most common molecular subtype of AML.[2,7] 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.[15,29,37] 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.[23,24,39] Observation of variant allele frequencies in relapsing disease and results of digital PCR assays show-

ing the persistence of *DNMT3A* and *IDH* mutations at high levels in patients in long-term remission indicate that preleukemic clones are frequently found in adults who have *NPM1*-mutated AML (Fig. S4 and S5 in Supplementary Appendix 1). Our data suggest that although chemotherapy can effectively target the mutated *NPM1* clone, coexisting *DNMT3A* R882H mutant populations are resistant but can be eliminated by stem-cell transplantation. However, a recent study identified *DNMT3A* mutations in follow-up samples from patients in very long-term remission, indicating that elimination may not be essential for cure.[40] Our findings showed that, regardless of clonal architecture, mutated *NPM1* provided a reliable marker of AML status in the majority of patients and that molecular relapse reliably predicted disease progression.

In conclusion, our study involving a large cohort of intensively treated patients showed that the presence of minimal residual disease predicted relapse and was superior to the baseline diagnostic molecular genetic markers that are currently used to guide decisions with respect to stem-cell transplantation. Although we did not find a significant benefit of transplantation in patients with minimal residual disease, the number of patients in the analysis was small, since only one third of the patients underwent transplantation within 3 months after a minimal-residual-disease sample was obtained following the second chemotherapy cycle. The question of whether outcomes might be improved by more rapid deployment of transplantation is being studied in the ongoing NCRI AML 19 trial (Current Controlled Trials number, ISRCTN78449203). Although it is recognized that the most informative sample source (peripheral blood or bone marrow) and time points may vary according to treatment protocol, these data lend support to a broadening of the scope of detection of minimal residual disease to assess response and to identify a group of patients with a poor prognosis who may be candidates for transplantation or new therapies.

The views expressed in this article are those of the authors and do not necessarily represent the official position of the National Health Service (NHS) or the National Institute for Health Research (NIHR).

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APPENDIX

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