VOLUME 31 · NUMBER 31 · NOVEMBER 1 2013

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

High Prognostic Impact of Flow Cytometric Minimal Residual Disease Detection in Acute Myeloid Leukemia: Data From the HOVON/SAKK AML 42A Study

Monique Terwijn, Wim L.J. van Putten, Angèle Kelder, Vincent H.J. van der Velden, Rik A. Brooimans, Thomas Pabst, Johan Maertens, Nancy Boeckx, Georgine E. de Greef, Peter J.M. Valk, Frank W.M.B. Preijers, Peter C. Huijgens, Angelika M. Dräger, Urs Schanz, Mojca Jongen-Lavrecic, Bart J. Biemond, Jakob R. Passweg, Michel van Gelder, Pierre Wijermans, Carlos Graux, Mario Bargetzi, Marie-Cecile Legdeur, Jurgen Kuball, Okke de Weerdt, Yves Chalandon, Urs Hess, Leo F. Verdonck, Jan W. Gratama, Yvonne J.M. Oussoren, Willemijn J. Scholten, Jennita Slomp, Alexander N. Snel, Marie-Christiane Vekemans, Bob Löwenberg, Gert J. Ossenkoppele, and Gerrit J. Schuurhuis

See accompanying editorial on page 3857

Author affiliations appear at the end of this article.

Published online ahead of print at www.jco.org on September 23, 2013.

Supported in part by Grant No. KWF 2006-3695 from the Dutch Cancer Society.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Gerrit J. Schuurhuis, PhD, VU University Medical Center, Department of Hematology, CCA-4.28, PO Box 7057, 1007 MB Amsterdam, the Netherlands; e-mail: gj.schuurhuis@vumc.nl.

0732-183X/13/3131w-3889w/$20.00

DOI: 10.1200/JCO.2012.45.9628

ABSTRACT

Purpose
Half the patients with acute myeloid leukemia (AML) who achieve complete remission (CR), ultimately relapse. Residual treatment-surviving leukemia is considered responsible for the outgrowth of AML. In many retrospective studies, detection of minimal residual disease (MRD) has been shown to enable identification of these poor-outcome patients by showing its independent prognostic impact. Most studies focus on molecular markers or analyze data in retrospect. This study establishes the value of immunophenotypically assessed MRD in the context of a multicenter clinical trial in adult AML with sample collection and analysis performed in a few specialized centers.

Patients and Methods
In adults (younger than age 60 years) with AML enrolled onto the Dutch-Belgian Hemato-Oncology Cooperative Group/Swiss Group for Clinical Cancer Research Acute Myeloid Leukemia 42A study, MRD was evaluated in bone marrow samples in CR (164 after induction cycle 1, 183 after cycle 2, 124 after consolidation therapy).

Results
After all courses of therapy, low MRD values distinguished patients with relatively favorable outcome from those with high relapse rate and adverse relapse-free and overall survival. In the whole patient group and in the subgroup with intermediate-risk cytogenetics, MRD was an independent prognostic factor. Multivariate analysis after cycle 2, when decisions about consolidation treatment have to be made, confirmed that high MRD values (> 0.1% of WBC) were associated with a higher risk of relapse after adjustment for consolidation treatment time-dependent covariate risk score and early or later CR.

Conclusion
In future treatment studies, risk stratification should be based not only on risk estimation assessed at diagnosis but also on MRD as a therapy-dependent prognostic factor.

J Clin Oncol 31:3889-3897. © 2013 by American Society of Clinical Oncology

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by an abnormal proliferation of myeloid progenitor cells and subsequent bone marrow (BM) failure. Despite high remission rates after intensive chemotherapy, 5-year survival is only approximately 30% to 40%. Apart from increasing complete remission (CR) rates, an important goal for treatment, guided by prognostic factors at diagnosis, is to tune clinical management in the postremission phase. Currently, the most important prognostic factors at diagnosis encompass cytogenetics and molecular abnormalities.[1-4] Although of utmost importance in risk stratification, treatment outcome for specifically defined risk groups is still highly variable, especially in intermediate-risk AML. Thus, there is a need for additional prognostic factors, which may include treatment- and response-related factors. In several correlative studies, minimal residual disease (MRD)

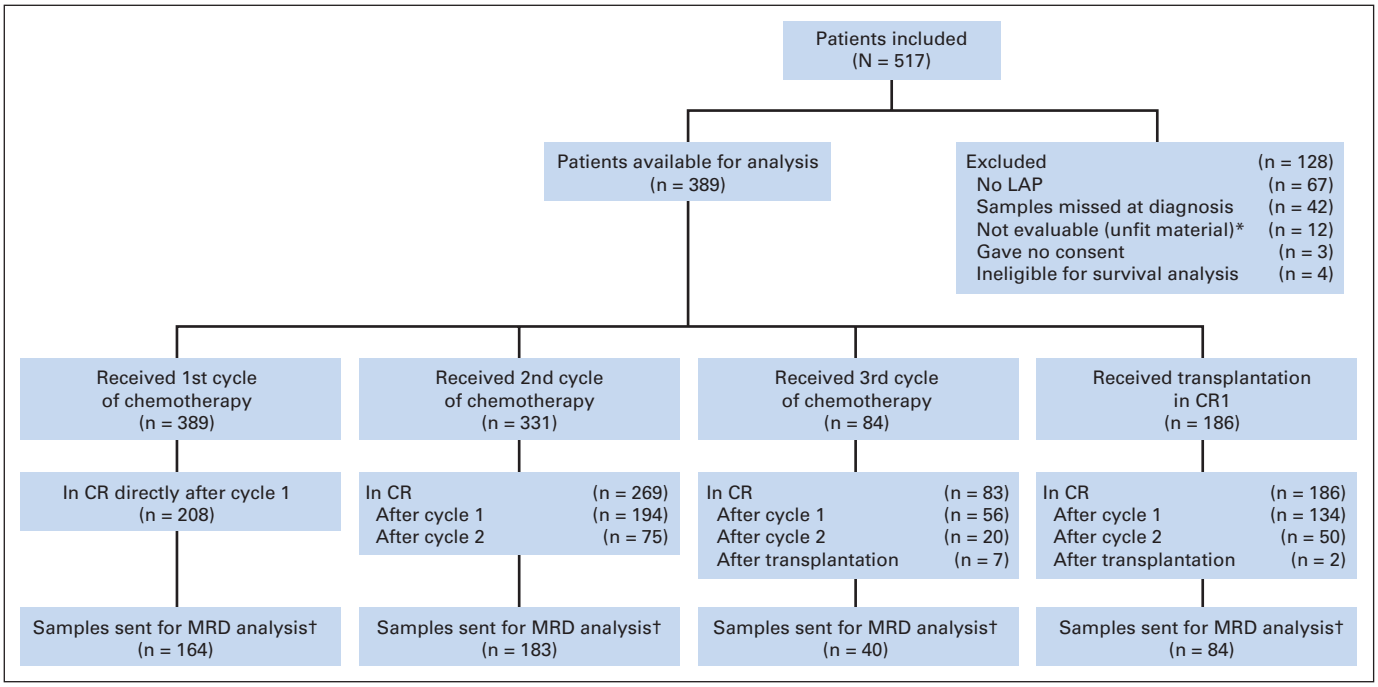

Fig 1. Diagram of patients included the HOVON/SAKK AML 42A study. Of 517 patients included, 389 patients showed one or more leukemia-associated phenotypes (LAPs) at diagnosis and were suitable for the monitoring of minimal residual disease (MRD) in remission bone marrow. Bone marrow samples from 164 patients were available for MRD analysis after the first cycle of chemotherapy, 183 samples were available after the second cycle, 40 samples were available after the third cycle, and 84 samples were available after transplantation. (*) Due to dry tap and no blasts in peripheral blood or poor quality material with mainly dead cells. (†) Drop-off could be only partly explained with death or relapse before the next cycle could be given or within 3 months after consolidation treatment (cycle 1, n = 0; cycle 2, n = 3; cycle 3, n = 13; transplantation, n = 11). Other missing samples were not received. CR, complete response; CR1, first CR.

has been convincingly shown to provide such additional prognostic information.[5-15] Of interest, in the study by Rubnitz et al,[16] MRD remained a prognostic factor in patients in whom treatment was intensified on the basis of MRD positivity. MRD is defined as leukemic cells persisting after chemotherapy below the sensitivity (detection limit) of routine morphology. The most widely used techniques to assess MRD in AML use molecular or immunophenotypic aberrancies. For the immunophenotypic identification of MRD, aberrantly expressed markers are combined with normal myeloid antigens and, when possible, progenitor markers, resulting in a so-called leukemia-associated phenotype (LAP), which must be established at diagnosis. Because the LAP is not present on normal cells (or is present at relatively low frequencies), remission BM can be analyzed for LAP-positive MRD cells with sensitivities ranging from 10^{-3} to 10^{-5} (one leukemic cell in 1,000 to 100,000 WBCs).[7,9,12,17,18] It is important to realize that these studies in patients with AML were performed retrospectively, mostly in a single-institute setting, thereby introducing well-known potential bias. By identifying cut point values on the order of 0.01% to 0.1%, it has been possible to identify patients markedly differing in prognosis. In our retrospective study, we showed similar results with cut points usable in a range of 0.06% to 1%.[10] In this study, we set out to prospectively validate the previously defined cut points in a multicenter international clinical trial (Dutch-Belgian Hemato-Oncology Cooperative Group/Swiss Group for Clinical Cancer Research Acute Myeloid Leukemia [HOVON/SAKK AML] 42A). To that end, we determined MRD percentages in a setting in which MRD assessment was performed without prior knowledge of clinical management, diagnostic features, or outcome. This study differs from other studies[6,7,10,12,19] since the patients were enrolled onto a large multicenter clinical study with preplanned sample collection and MRD analysis in a few specialized centers according to common protocols. The results show that by using methods established earlier, the previously defined cut points[10] are highly predictive for clinical outcome.

PATIENTS AND METHODS

The Data Supplement provides more detailed information.

Patients and Treatment

A total of 517 patients between the ages of 18 and 60 years were included in this study (Fig 1). Patients were randomly assigned to receive granulocyte colony-stimulating factor (G-CSF; 5 μg/kg) or no G-CSF during induction treatment. Data for these two groups were pooled since clinically there is no difference in survival.[20] In agreement with this, no significant differences were found between groups in MRD percentage after all therapy cycles. Patients were assigned to three risk groups on the basis of the following criteria: (1) good-risk patients included those positive for t(8;21) with WBC $\leq 20 \times 10^9$/L, (2) those with inv(16) or t(16;16) and (3) those without a monosomal karyotype but with mutated *CEBPα* and those with mutated *NPM1/FLT3* wild-type in CR after the first induction cycle. Poor-risk patients were defined as having non–core-binding factor leukemia with a monosomal karyotype, being positive for *EVI1*, or having 3q26 abnormalities. The remaining patients were classified as having intermediate-risk disease. Patient and treatment characteristics are provided in Table 1.

Sampling and Logistics

Thirty-one centers participated in collection of patient samples as a part of the MRD side study of the HOVON/SAKK AML 42A clinical study. LAP assessment and MRD analysis were performed in four centers. A summary of

Table 1. Patient and Treatment Characteristics

Characteristic	No. of Patients at Diagnosis	%	No. of Patients With MRD ≤ 0.1% After Cycle 2	%	No. of Patients With MRD > 0.1% After Cycle 2	%
Total	241*		141		42	
Sex						
Male	122	51	73		24	
Female	119	49	68		18	
Age, years						
Median	48		48		43	
Range	18-60		18-60		21-58	
≤ 40	71	29	44	31	17	40
> 40	170	71	97	69	25	60
WBC at diagnosis ($\times 10^9$/L)						
≤ 20	136	56	87	62	18	43
20-100	69	29	42	30	10	24
> 100	36	15	12	9	14	33
AML type						
De novo AML	203	84	121	86	34	81
Secondary AML	21	9	8	6	7	17
RAEB	6	2	5	4	1	2
RAEB-t	11	5	7	5	0	0
Consolidation treatment						
None	32	13	15	11	6	14
Cycle 3	52	22	26	18	14	33
Autologous SCT	65	27	44	31	10	24
Allogeneic SCT	92	38	56	40	12	29
Risk group						
Good	64	27	38	27	14	33
Intermediate	143	59	88	62	19	45
Poor	34	14	15	11	9	21
CR achieved						
After cycle 1	181	75	114	81	23	55
After cycle 2	60	25	27	19	19	45
G-CSF						
Did not receive G-CSF	115	48	64	45	19	45
Received G-CSF	126	52	77	55	23	55

Abbreviations: AML, acute myeloid leukemia; CR, complete response; G-CSF, granulocyte colony-stimulating factor; MRD, minimal residual disease; RAEB, refractory anemia with excess blasts; RAEB-t, RAEB in transformation; SCT, stem-cell transplantation.

*Total No. of patients available for MRD analysis in whom at least one sample was used for MRD analysis in landmark cycle 1, landmark cycle 2, or consolidation.

the logistics of sampling and MRD analysis is provided in the Data Supplement. BM samples were collected at diagnosis and at follow-up after every cycle of chemotherapy. LAP assessment and MRD analysis were performed in a setting in which the laboratories had no access to patients' clinical data until final MRD data were sent to the statistician. In addition, clinicians had no access to the MRD status of their patients. The data were included only for patients with a morphologic CR. Figure 1 shows the details of sampling outcome.

LAP Assessment

LAP assessment was performed in collaboration with the Dutch-Belgian MRD flow cytometry taskforce and was done in a two-step procedure. As a first step, a standard screening panel was designed to assess the immunophenotype of blasts, identified as dim expression of CD45 with low sideward scatter properties (Data Supplement). The second step consisted of the validation of the composed LAP by showing its actual presence on the leukemic cells, which should be on at least 10% of the blast population. The Data Supplement gives an overview of different LAPs identified in the study. The Data Supplement also shows LAP types as detected at diagnosis as well as LAPs actually used in follow-up for MRD assessment. For refractory anemia with excess blasts in transformation, only LAPs that covered the blast fraction were defined; here, no LAPs present on mature cells were used. Information on clones and commercial sources of all monoclonal antibodies used is provided in the Data Supplement.

MRD Detection

MRD analysis was performed as previously described.[10] Analysis of LAP-positive cells included multiple backgating steps to ensure that, compared with diagnosis, the LAP-positive cells show fairly identical positions in forward scatter channel/side scatter channel and CD45 expression. By using this method, LAP-positive cell populations could be distinguished from background expression in the gate. MRD percentage was defined as the percentage of LAP-positive cells within the WBC compartment multiplied by the correction factor: 100%/percentage of LAP-positive blasts at diagnosis. When there was a considerable amount of background expression in the gate, the correction factor[10] was set to 1. In addition, calculations of MRD percentage have also been performed by using uncorrected LAP-positive frequencies, both as percentage of WBC and as log reduction of LAP-positive cells (in both cases, LAP-positive events are relative to WBC count). MRD was also defined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)–based log reduction. Total RNA was extracted, and complementary DNA (cDNA) was synthesized from 500 ng of RNA by using random hexamer priming, essentially as described.[21]

Statistical Analysis

Separate analyses were performed for three landmarks: sample in CR after cycle 1, after cycle 2, and after consolidation treatment. Primary end point for all analyses was relapse with censoring at death in first CR (CR1). Secondary end points were relapse-free survival (RFS), in which death in CR1 was included as a competing risk event, and overall survival (OS). In each landmark analysis, time was measured from the date of sampling. RFS, OS, and relapse incidence curves[22] were calculated according to Kaplan-Meier. In addition, competing risks actuarial estimates of relapse and death in CR1 at 4 years were estimated by cumulative incidence functions[23] and are presented in Table 2.

RESULTS

Regression Analysis for Corrected and Uncorrected MRD Percentage and LAP-Positive Log Reduction

MRD percentages were assessed by including a correction factor as described in Patients and Methods. In addition, we investigated the prognostic impact of the percentage of LAP-positive cells without a correction factor and the log reduction of LAP-positive cells, an approach previously used by Kern et al.[11] For each of the three covariates—log-transformed percentage of MRD,[10] log-transformed percentage of LAP-positive cells, and LAP-positive cell log reduction—Cox regression analysis after landmark cycle 2 with the end point of relapse was done with adjustment for AML risk and early or late CR. All three covariates showed a highly significant association with risk of relapse: hazard ratio (HR), 1.49 ($P = .007$); HR, 1.50 ($P = .015$); and HR, 0.66 ($P = .009$), respectively. Note that a high log reduction of LAP-positive cells corresponds with a low percentage of LAP-positive cells. After landmark consolidation, similar results were found: HR, 3.2 ($P = 3.0 \times 10^{-8}$); HR, 3.8 ($P = 1.6 \times 10^{-6}$); and HR, 0.38 ($P = 7 \times 10^{-6}$), respectively.

To validate our previous results (described in the following paragraphs), further analyses were performed, mainly with corrected MRD percentages.[10] The highly significant association seen between MRD percentage as a continuous covariate and the risk of relapse allows searching for optimal cut points established in a wide range

Table 2. Univariate and Multivariate Cox Regression Analysis of Relapse Incidence for Various Prognostic Factors, Including MRD After Landmark Cycle 2 in AML

Variable	No. of Patients	Relapse and Death in CR1					Univariate Analysis			Multivariate Analysis		
		No. of Patients Who Relapsed	No. of Deaths in CR1	RFS % at 4 Years*	Relapse % at 4 Years*	Death in CR1 % at 4 Years*	HR	95% CI	*P*	HR	95% CI	*P*
Total	183	82	11	46	48	6						
MRD after cycle 2									< .001			.001
MRD ≤ 0.1%	141	53	9	52	42	7						
MRD > 0.1%	42	29	2	23	72	5	2.97	1.88 to 4.70		2.60	1.49 to 4.55	
AML type									.003			.054
De novo	155	63	11	50	43	7						
Secondary	15	12	0	13	87	0	3.47	1.86 to 6.47		2.68	1.33 to 5.42	
MDS	13	7	0	27	73	0	1.14	0.52 to 2.48		0.65	0.27 to 1.56	
CR achieved									< .001			.021
After cycle 1	137	52	9	50	43	7						
After cycle 2	46	30	2	30	65	4	2.44	1.55 to 3.84		1.67	1.00 to 2.79	
Risk of AML									.001			< .001
Good	52	16	1	61	37	2						
Intermediate	107	50	8	43	49	8	1.81	1.03 to 3.18		2.55	1.32 to 4.94	
Poor	24	16	2	25	67	8	3.75	1.87 to 7.54		4.75	2.08 to 10.83	
WBC at diagnosis ($\times 10^9$/L)									.005			.0026
≤ 100	157	65	10	48	45	7						
> 100	26	17	1	31	65	4	2.31	1.35 to 3.95		2.12	1.12 to 4.01	
Age, years									.13			.084
≤ 40	61	22	4	56	37	7						
> 40	122	60	7	40	54	6	1.44	0.89 to 2.35		1.56	0.93 to 2.63	
G-CSF									.11			.084
G-CSF ≤ 0.1%	83	42	6	35	57	7						
G-CSF > 0.1%	100	40	5	53	42	5	0.70	0.45 to 1.08		0.66	0.42 to 1.06	
Last consolidation treatment									< .001			< .001
None	21	19	0	0	100	0						
Cycle 3	40	23	0	33	67	0	0.46	0.25 to 0.87		0.52	0.25 to 1.10	
Autologous SCT	54	19	2	63	33	4	0.23	0.12 to 0.46		0.19	0.09 to 0.41	
Allogeneic SCT	68	21	9	54	33	13	0.23	0.12 to 0.44		0.19	0.09 to 0.40	

NOTE. In the univariate analysis, results are given for the relapse end point for each prognostic factor separately. In the multivariate analysis, all variables shown in the left-most column were included. Consolidation treatment was included in the regression analyses with time-dependent covariates.

Abbreviations: AML, acute myeloid leukemia; CR, complete response; CR1, first CR; G-CSF, granulocyte colony-stimulating factor; HR, hazard ratio; MDS, myelodysplastic syndrome; MRD, minimal residual disease; RFS, relapse-free survival; SCT, stem-cell transplantation.

*Actuarial estimates at 4 years of RFS and cumulative incidence of competing risks relapse and death in CR1.

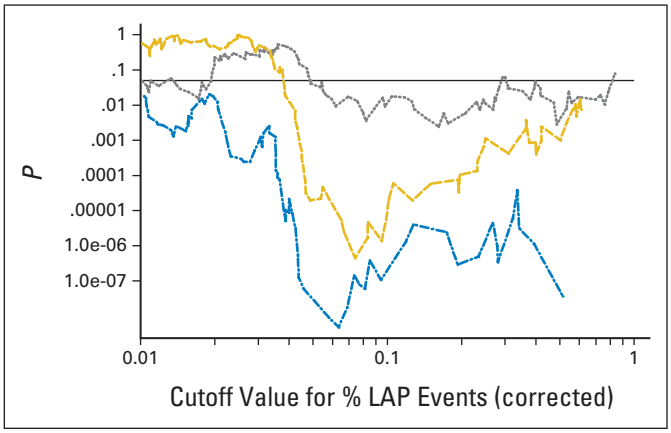

Fig 2. *P* values for various minimal residual disease cut points. *P* values for relapse by cut points for the percentage of minimal residual disease (MRD)–positive patients (using corrected leukemia-associated phenotypes (LAP)–positive values, as outlined in Patients and Methods), for three landmark analyses: after first cycle (gray dotted line; n = 164), second cycle (gold dashed line; n = 183), and after consolidation therapy (blue dotted-dashed line; n = 121). For each cut point, the group of patients with values above the cut point were compared with those below the cut point by using Cox regression analysis with end point relapse, with adjustment for risk group, after cycle 2, after consolidation, and for late versus early CR. The horizontal line represents the *P* value of .05, which is considered the borderline for statistical significance.

of MRD values (between 0.01%, which is the detection limit, and 1%) and discriminating patients with high MRD (MRD-positive, poor prognosis) from patients with low MRD (MRD-negative, good prognosis).

Landmark Analyses After Cycle 1, Cycle 2, and Consolidation Treatment

After remission induction cycle 1, median MRD was 0.040% (range, 0.01% to 16%; n = 164). The dotted curve in Figure 2 shows *P* values for a test of difference in relapse rates between MRD-positive patients and MRD-negative patients in the MRD range of 0.01% to 1%. For almost all cut points between 0.05% and 0.8%, differences were significant ($P < .05$).

After remission induction cycle 2, median MRD was 0.023% (range, 0.01% to 21%; n = 183). The dashed line in Figure 2 shows *P* values in the MRD range of 0.01% to 1%. For all cut points more than 0.04%, differences were significant.

After consolidation treatment, 121 patients were evaluable, of whom 32 had received chemotherapy only, 44 had received autologous stem-cell transplantation, and 45 had received allogeneic stem-cell transplantation. Median MRD was 0.021% (range, 0.01% to 9.6%). The dotted-dashed line in Figure 2 shows *P* values in the MRD range of 0.01% to 1%. For all cut points greater than 0.01%, differences were significant. The same analysis for uncorrected LAP-positive percentage, LAP-positive log reduction, and analysis with competing risk regression[24] shows similar results after all cycles (Data Supplement).

These analyses validate our retrospectively defined cut points,[10] although the cut points suggested by others are within the ranges described earlier.[25] With the limited number of patients per landmark, it is not possible to accurately determine the optimal cut point, which is also reflected by the fluctuation in *P* values shown in Figure 2 (and the Data Supplement). For reasons of applicability in the clinic and uniformity after every cycle of chemotherapy, we decided to use a cut point of 0.1% to distinguish MRD-negative patients (MRD-negative, $\leq 0.1\%$) from MRD-positive patients (MRD-positive, $> 0.1\%$). The upper panel in Figure 3 shows that relapse incidence is higher for MRD-positive versus MRD-negative patients for the three landmarks. In addition, MRD-positive patients showed adverse outcome after the three cycles: for OS, $P = .03$, $P < .001$, and $P = .008$, respectively; for RFS, $P = .008$, $P = .001$, and $P < .001$, respectively (Data Supplement).

Proportional Hazard Is Highest in the First Year

The upper-middle and upper-right panels of Figure 3 suggest nonproportionality, with the largest differences in the rates of relapse seen in the first 12 months, without additional differences at longer follow-up. We have tested this by estimating the HRs for relapse of MRD-positive versus MRD-negative patients after landmark cycle 2 (adjusted for CR, AML risk, WBC, consolidation treatment, age, and G-CSF) in the periods 0 to 6 months: 35 relapses; HR, 13.0 [95% CI, 5.3 to 32.1]; 7 to 12 months: 26 relapses; HR, 4.7 [95% CI, 2.1 to 10.5]; and more than 12 months: 21 relapses; HR, 0.7 [95% CI, 0.2 to 2.4].

The difference between the three hazard ratios is highly statistically significant (likelihood ratio test $\chi^2 = 20.9$ with 2 *df*; $P < .001$). This clearly shows that the MRD status is a strong prognostic indicator for the risk of relapse only in the first year.

Use of MRD for Risk-Based Decisions About Consolidation Treatment

After induction treatment, decisions about consolidation treatment have to be made for patients in CR. In the HOVON/SAKK AML 42A study, these decisions were based on traditional risk parameters such as early or late CR and cytogenetics. Because MRD information was not available at that time, we performed multivariate Cox analysis after cycle 2 to see how knowledge of the MRD status could add to the information on prognostic factors determined at diagnosis (Table 2; additional statistical information in the Data Supplement). Twenty-one patients did not receive any consolidation treatment, of whom 19 have relapsed. For most of these patients, poor condition, slow recovery after cycle 2, and (early) relapse prevented them from getting consolidation treatment. The estimated HR for MRD-positive compared with MRD-negative patients is almost the same in both analyses (2.97 in univariate and 2.60 in multivariate analysis). Notice that these values are higher than the HRs for late CR (2.44 and 1.67, respectively), WBC more than 100×10^9/L (2.31 and 2.12×10^9/L, respectively), and intermediate risk (1.81 and 2.55, respectively) with respect to good risk. Within each of the three risk groups, the MRD-positive patients showed a significantly higher risk of relapse (Figs 3D to 3F).

Relation Between MRD After Cycle 2 and Clinical and Molecular Subgroups

Subgroup analyses were performed for MRD after cycle 2 with various clinical and molecular parameters (Data Supplement). Because of the relatively small groups, the results should be considered exploratory. First, for each parameter, we looked for differences between the subgroups in percentages of MRD-positive patients. The overall percentage of MRD-positive patients after cycle 2 was 23%. A significantly higher proportion was found for patients with late CR (19 [41%] of 46; $P = .001$), a lower proportion for *NMP1*-positive patients (five [9%] of 54; $P = .01$), and an even lower proportion for *FLT3-ITD*–negative/*NPM1*-positive patients (one [3%] of 30; $P = .01$). For the other

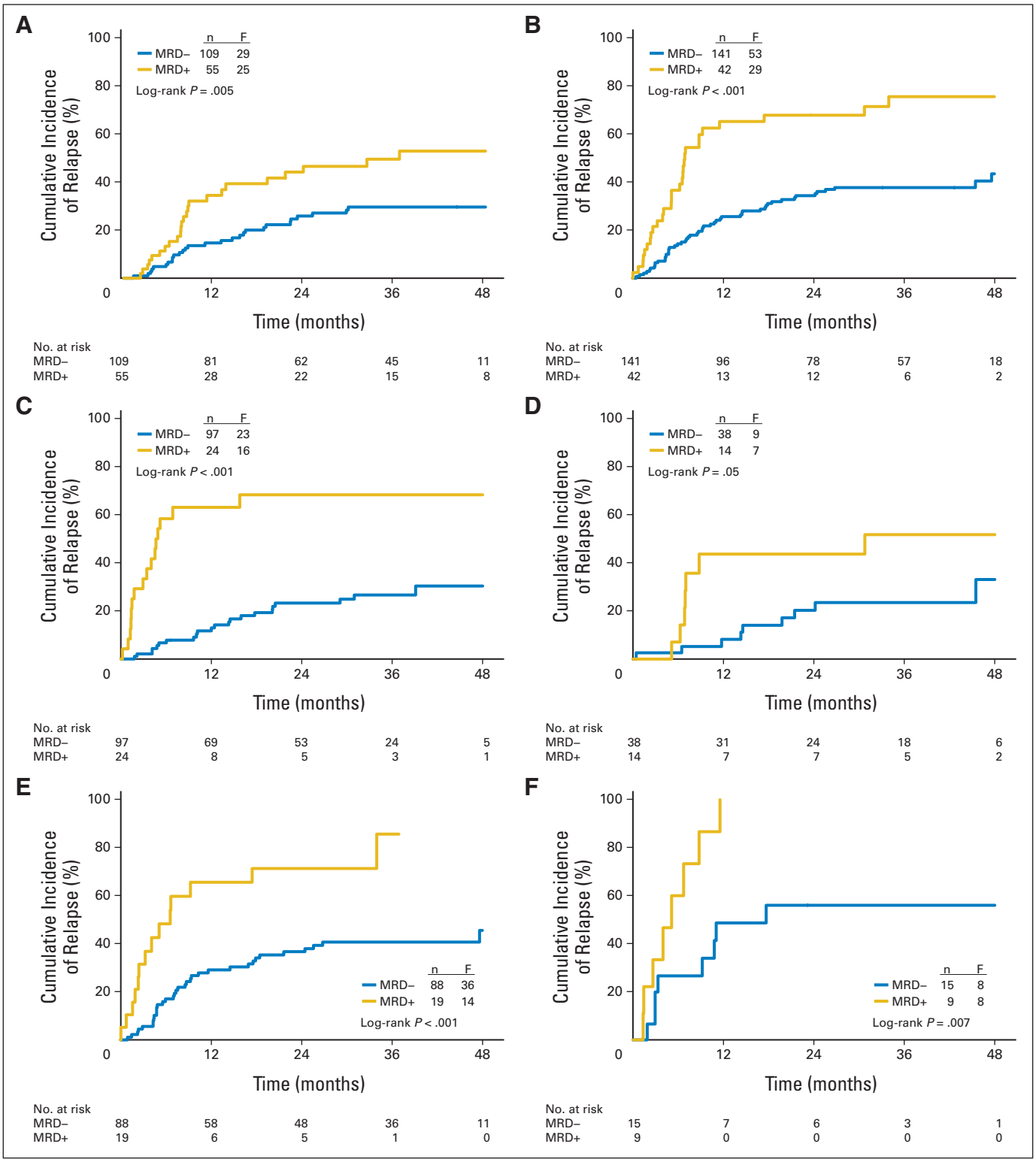

Fig 3. Relapse incidence (RI) by minimal residual disease (MRD). RI curves for complete response (A) after induction cycle 1, (B) after cycle 2, and (C) after consolidation treatment. RI curves for risk classification (D) after cycle 2, good risk; (E) after cycle 2, intermediate risk; and (F) after cycle 2, poor risk. F, failure; MRD-, MRD ≤ 0.1%; MRD+, MRD > 0.1%.

variables, no significant differences were found. Subsequently, we tested for differences between subgroups of each parameter in the HR for relapse of MRD-positive compared with MRD-negative patients. The HR for MRD-positive patients with late CR was much higher (HR, 6.76) than that for patients with CR after cycle 1 (HR, 1.42; test for interaction $P = .006$). This is illustrated in Figure 4A. The 19 patients who were MRD-positive with late CR after induction cycle 2 showed the most adverse outcome ($P < .001$), which is independent of AML risk group.

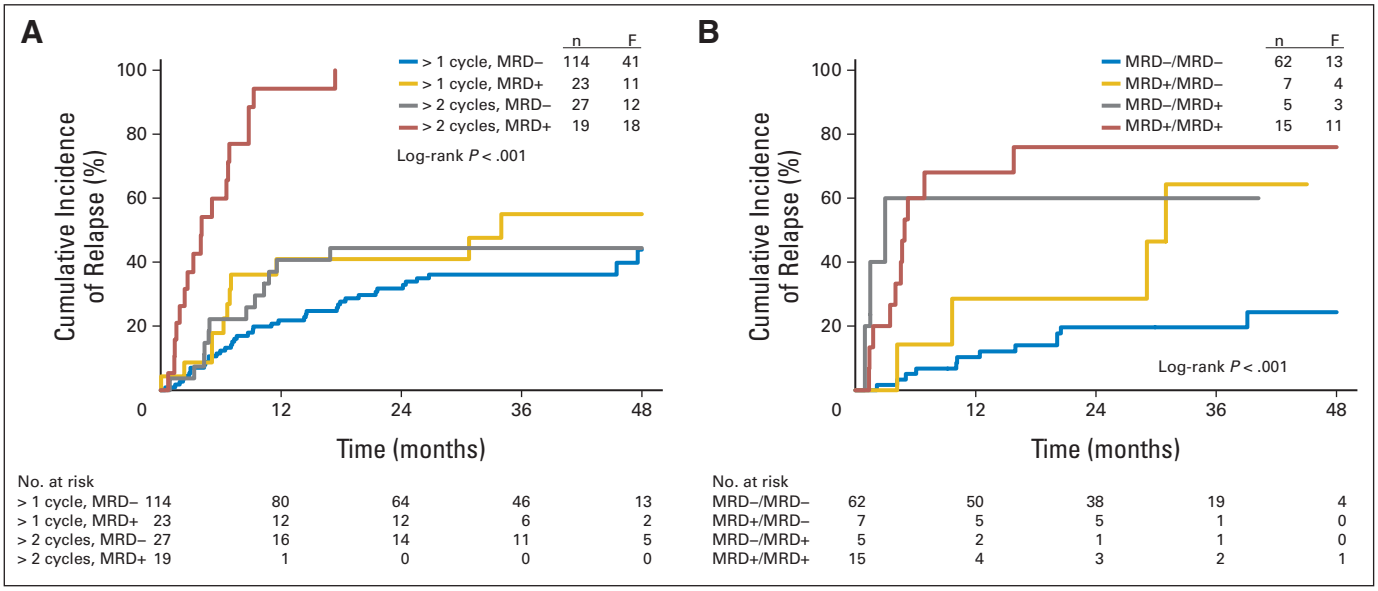

Fig 4. Relapse incidence by minimal residual disease (MRD). (A) After cycle 2, split by complete response reached after cycle 1 or 2 and combined with MRD status after cycle 2. (B) After consolidation treatment, split by MRD status after induction cycle 2. F, failure; MRD-, MRD ≤ 0.1%; MRD+, MRD > 0.1%.

Outcome When Combining MRD Status After Cycle 2 and Consolidation Therapy

In 89 patients, MRD data were available after both induction cycle 2 and consolidation therapy. For most of these patients, the MRD status was the same after cycle 2 and after consolidation (negative/negative, n = 62; positive/positive, n = 15). Twelve patients showed different MRD status (positive/negative, n = 7; negative/positive, n = 5). Figure 4B shows the relapse incidence after consolidation in the four subgroups. Multivariate analysis after consolidation did not show a better fit by inclusion of the MRD status after cycle 2 in the model, but this is not surprising, given high concordance between MRD status after cycle 2 and consolidation and the small numbers in the subgroups.

Comparative MRD Assessed by Flow Cytometry and qRT-PCR

In addition to MRD detection based on immunophenotyping by flow cytometry, we also determined MRD defined by qRT-PCR for *NPM1* mutation, *AML1-ETO*, and *CBFB-MYH1*, thereby realizing that this represents a subgroup with relatively good prognosis. We compared log reduction by qRT-PCR with log reduction of LAP-positive cells for both by using the optimal cut point of 2.4, primarily assessed for qRT-PCR, but which is also within the range of optimal cut points shown for flow cytometry in the Data Supplement. Both approaches correlate moderately well: 71%, with discrepancies found in 29% of the patients (qRT-PCR^{high}/flow^{low} in 33 of 141 patients and qRT-PCR^{low}/flow^{high} in eight of 141 patients; Spearman Rank correlation $\rho = 0.22$; $P = .008$).

DISCUSSION

Several retrospective studies of the clinical value of immunophenotypic MRD detection in adult AML have been reported in the last decade, including a study at one of the HOVON/SAKK centers: Vrije Universiteit Medical Centre in Amsterdam, the Netherlands.[10] In this study and those of others, MRD percentage was found to be an independent prognostic factor for OS and RFS when assessed in a postinduction or postconsolidation single-center setting. To the best of our knowledge, our study is the first in which retrospectively defined cut points have been validated in a prospective cohort by using patient samples from a large multicenter clinical trial. Here, we confirm that all previously defined cut points hold up and effectively distinguish patients with adverse prognosis from those with good prognosis for the whole patient group. From a clinical practice viewpoint, MRD assessment is perhaps most useful in patients with intermediate prognostic risk because they most likely still represent a mix of variable risk subcategories, with large heterogeneity in treatment outcome. In this intermediate-risk group, too, MRD had independent prognostic value. This shows that flow cytometric detection of MRD in AML offers a powerful tool that can be used prospectively for clinical decision making. On the basis of this observation, we suggest that patients in the cytogenetic and/or molecular intermediate-risk group who are MRD positive with a late CR after cycle 2 should be treated as if they were poor risk. To determine the optimal postremission therapy for patients with AML, MRD assessment offers a novel postdiagnosis treatment-related therapy stratification. To guide risk-based treatment for the individual patient, an early evaluation of MRD percentage (after induction therapy) is essential. In that respect, the finding that MRD status after consolidation treatment overrules MRD status after second induction cycle confirms the findings of Maurillo et al[6] and may have no practical consequences. For example, that time point will be too late to decide on allo-STC. In addition, subgroup analysis revealed that patients achieving a late CR together with MRD positivity after cycle 2 proved to have extremely poor outcome. Since these data should be considered exploratory, it would be of interest to learn whether this finding could be confirmed by others. If so, this group especially qualifies for exploring new avenues of treatment such as post-transplantation epigenetic modulation, which will be tested in a

new HOVON study. Although it is possible to detect MRD by flow cytometry at a sensitivity of 1 in 100,000 cells (0.001%),[18] the maximum sensitivity achievable in most patients is between 0.01% and 0.1%. We adopted the cut point of 0.1% to define MRD-positive patients in this study because of its uniformity and applicability after all cycles of treatment. MRD assessed after cycle 2 presents itself as an independent adverse prognostic factor that can be used to distinguish poor risk in 23% of the evaluable patients.

Despite the prognostic relevance of MRD monitoring, independent of the cut point used, a portion of the MRD-negative patients still relapse. Since it has been hypothesized that leukemic stem cells are primarily responsible for relapses in AML, monitoring residual leukemic stem cells, as previously described,[26,27] in addition to monitoring MRD might lead to a more accurate prediction of survival outcome. However, despite higher specificity and less subjectivity in assessment,[25-27] the sensitivity of this method is lower because of the small size of the stem-cell compartment.[28]

In summary, this study shows that MRD positivity predicts adverse clinical outcome in AML after induction treatment as well as after consolidation therapy and may now allow improved patient-tailored risk-based therapy.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: None **Consultant or Advisory Role:** None **Stock Ownership:** Peter J.M. Valk, Skyline Diagnostics Rotterdam **Honoraria:** None **Research Funding:** None **Expert Testimony:** None **Patents:** None **Other Remuneration:** None

AUTHOR CONTRIBUTIONS

Conception and design: Monique Terwijn, Vincent H.J. van der Velden, Rik A. Brooimans, Peter C. Huijgens, Nancy Boeckx, Urs Schanz, Frank W.M.B. Preijers, Johan Maertens, Bob Löwenberg, Gert J. Ossenkoppele, Gerrit J. Schuurhuis

Administrative support: Angelika M. Dräger, Yvonne J.M. Oussoren, Frank W.M.B. Preijers, Willemijn J. Scholten, Jennita Slomp, Johan Maertens

Provision of study materials or patients: Vincent H.J. van der Velden, Rik A. Brooimans, Peter C. Huijgens, Nancy Boeckx, Thomas Pabst, Urs Schanz, Georgine E. de Greef, Mojca Jongen-Lavrecic, Bart J. Biemond, Jakob R. Passweg, Michel van Gelder, Pierre Wijermans, Carlos Graux, Mario Bargetzi, Marie-Cecile Legdeur, Jurgen Kuball, Okke de Weerdt, Yves Chalandon, Urs Hess, Jan W. Gratama, Leo F. Verdonck, Marie-Christiane Vekemans, Johan Maertens, Bob Löwenberg, Gert J. Ossenkoppele

Collection and assembly of data: Monique Terwijn, Wim L.J. van Putten, Angèle Kelder, Vincent H.J. van der Velden, Rik A. Brooimans, Nancy Boeckx, Thomas Pabst, Georgine E. de Greef, Mojca Jongen-Lavrecic, Bart J. Biemond, Jakob R. Passweg, Michel van Gelder, Pierre Wijermans, Carlos Graux, Mario Bargetzi, Marie-Cecile Legdeur, Jurgen Kuball, Okke de Weerdt, Yves Chalandon, Urs Hess, Jan W. Gratama, Yvonne J.M. Oussoren, Frank W.M.B. Preijers, Leo F. Verdonck, Willemijn J. Scholten, Jennita Slomp, Alexander N. Snel, Peter J.M. Valk, Marie-Christiane Vekemans, Johan Maertens, Gerrit J. Schuurhuis

Data analysis and interpretation: Monique Terwijn, Wim L.J. van Putten, Angèle Kelder, Vincent H.J. van der Velden, Rik A. Brooimans, Peter C. Huijgens, Nancy Boeckx, Thomas Pabst, Angelika M. Dräger, Mojca Jongen-Lavrecic, Yvonne J.M. Oussoren, Frank W.M.B. Preijers, Leo F. Verdonck, Willemijn J. Scholten, Jennita Slomp, Alexander N. Snel, Peter J.M. Valk, Johan Maertens, Bob Löwenberg, Gert J. Ossenkoppele, Gerrit J. Schuurhuis

Manuscript writing: All authors

Final approval of manuscript: All authors

REFERENCES

1. Grimwade D, Walker H, Oliver F, et al: The importance of diagnostic cytogenetics on outcome in AML: Analysis of 1,612 patients entered into the MRC AML 10 trial—The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood 92:2322-2333, 1998
2. Döhner H: Implication of the molecular characterization of acute myeloid leukemia. Hematology Am Soc Hematol Educ Program 2007:412-419, 2007
3. Grimwade D, Hills RK: Independent prognostic factors for AML outcome. Hematology Am Soc Hematol Educ Program 2009:385-395, 2009
4. Valk PJ, Verhaak RG, Beijen MA, et al: Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med 350:1617-1628, 2004
5. Kern W, Haferlach C, Haferlach T, et al: Monitoring of minimal residual disease in acute myeloid leukemia. Cancer 112:4-16, 2008
6. Maurillo L, Buccisano F, Del Principe MI, et al: Toward optimization of postremission therapy for residual disease-positive patients with acute myeloid leukemia. J Clin Oncol 26:4944-4951, 2008
7. San Miguel JF, Martínez A, Macedo A, et al: Immunophenotyping investigation of minimal residual disease is a useful approach for predicting relapse in acute myeloid leukemia patients. Blood 90:2465-2470, 1997
8. van der Velden VH, van der Sluijs-Geling A, Gibson BE, et al: Clinical significance of flowcytometric minimal residual disease detection in pediatric acute myeloid leukemia patients treated according to the DCOG ANLL97/MRC AML12 protocol. Leukemia 24:1599-1606, 2010
9. Vidriales MB, San-Miguel JF, Orfao A, et al: Minimal residual disease monitoring by flow cytometry. Best Pract Res Clin Haematol 16:599-612, 2003
10. Feller N, van der Pol MA, van Stijn A, et al: MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. Leukemia 18:1380-1390, 2004
11. Kern W, Voskova D, Schoch C, et al: Determination of relapse risk based on assessment of minimal residual disease during complete remission by multiparameter flow cytometry in unselected patients with acute myeloid leukemia. Blood 104:3078-3085, 2004
12. Venditti A, Buccisano F, Del Poeta G, et al: Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. Blood 96:3948-3952, 2000
13. Sievers EL, Lange BJ, Alonzo TA, et al: Immunophenotypic evidence of leukemia after induction therapy predicts relapse: Results from a prospective Children's Cancer Group study of 252 patients with acute myeloid leukemia. Blood 101:3398-3406, 2003
14. Coustan-Smith E, Ribeiro RC, Rubnitz JE, et al: Clinical significance of residual disease during treatment in childhood acute myeloid leukaemia. Br J Haematol 123:243-252, 2003
15. MRD-AML-BFM Study Group, Langebrake C, Creutzig U, et al: Residual disease monitoring in childhood acute myeloid leukemia by multiparameter flow cytometry: The MRD-AML-BFM Study Group. J Clin Oncol 24:3686-3692, 2006
16. Rubnitz JE, Inaba H, Dahl G, et al: Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: Results of the AML02 multicentre trial. Lancet Oncol 11:543-552, 2010
17. Buccisano F, Maurillo L, Spagnoli A, et al: Monitoring of minimal residual disease in acute myeloid leukemia. Curr Opin Oncol 21:582-588, 2009
18. Campana D: Status of minimal residual disease testing in childhood haematological malignancies. Br J Haematol 143:481-489, 2008

19. San Miguel JF, Vidriales MB, López-Berges C, et al: Early immunophenotypical evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups and may contribute to postinduction treatment stratification. Blood 98:1746-1751, 2001

20. Pabst T, Vellenga E, van Putten W, et al: Favorable effect of priming with granulocyte colony-stimulating factor in remission induction of acute myeloid leukemia restricted to dose escalation of cytarabine. Blood 119:5367-5373, 2012

21. Van der Reijden BA, de Wit L, van der Poel S, et al: Identification of a novel CBFB-MYH11 transcript: Implications for RT-PCR diagnosis. Hematol J 2:206-209, 2001

22. Labopin M, Latouche A, Suciu S, et al: Definitions and evaluation of endpoints following stem cells transplantation - Recommendation from the European group for Blood and Marrow transplantation (EBMT). Presented at the 35th Annual Meeting of the European Group for Blood and Marrow Transplantation, Göteborg, Sweden, March 29-April 1, 2009

23. Kim HT. Cumulative incidence in competing risks data and competing risks regression analysis. Clin Cancer Res 13:559-565, 2007

24. Fine JP, Gray RJ: A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc 94:496-509, 1999

25. Ossenkoppele GJ, van de Loosdrecht AA, Schuurhuis GJ: Review of the relevance of aberrant antigen expression by flow cytometry in myeloid neoplasms. Br J Haematol 153:421-436, 2011

26. van Rhenen A, van Dongen GA, Kelder A, et al: The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. Blood 110:2659-2666, 2007

27. van Rhenen A, Moshaver B, Kelder A, et al: Aberrant marker expression patterns on the CD34+ CD38- stem cell compartment in acute myeloid leukemia allows to distinguish the malignant from the normal stem cell compartment both at diagnosis and in remission. Leukemia 21:1700-1707, 2007

28. van Rhenen A, Feller N, Kelder A, et al: High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. Clin Cancer Res 11:6520-6527, 2005

Affiliations

Monique Terwijn, Angèle Kelder, Peter C. Huijgens, Angelika M. Dräger, Yvonne J.M. Oussoren, Willemijn J. Scholten, Alexander N. Snel, Gert J. Ossenkoppele, and Gerrit J. Schuurhuis, VU University Medical Centre; Bart J. Biemond, Academic Medical Centre, Amsterdam; Wim L.J. van Putten, Vincent H.J. van der Velden, Georgine E. de Greef, Peter J.M. Valk, Mojca Jongen-Lavrecic, and Bob Löwenberg, Erasmus University Medical Centre; Rik A. Brooimans, Jan W. Gratama, Erasmus University Medical Centre/Daniel den Hoed Cancer Centre, Rotterdam; Frank W.M.B. Preijers, Radboud University Nijmegen Medical Center, Nijmegen; Michel van Gelder, University Medical Centre, Maastricht; Pierre Wijermans, Haga Hospital, The Hague; Marie-Cecile Legdeur and Jennita Slomp, Medisch Spectrum Twente, Enschede; Jurgen Kuball, University Medical Center Utrecht; Okke de Weerdt, St. Antonius Hospital, Nieuwegein; Leo F. Verdonck, Isala Clinics, Zwolle, the Netherlands; Thomas Pabst, Bern University Hospital, Bern; Urs Schanz, University Hospital, Zürich; Jakob R. Passweg, Basel University Hospital, Basel; Mario Bargetzi, Kantonspital, Aarau; Yves Chalandon, University Hospital of Geneva, Geneva; Urs Hess, Kantonsspital, St. Gallen, Switzerland; Johan Maertens, University Hospital Gasthuisberg; Nancy Boeckx, University Hospitals Leuven and Katholieke Universiteit Leuven, Leuven; Carlos Graux, Cliniques Universitaires-Université Catholique de Louvain, Mont-Godinne, Yvoir; and Marie-Christiane Vekemans, St. Luc Hospital, Brussels, Belgium.

Acknowledgment

We acknowledge D. Velthuizen, J. te Marvelde, and H. Wind for their technical assistance. All clinicians and coordinating persons from the laboratories of the HOVON/SAKK centers are acknowledged for their invaluable contributions.