Prognostic impact of early response to induction therapy as assessed by multiparameter flow cytometry in acute myeloid leukemia

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A B S T R A C T

Background and Objectives. Early response after induction therapy is an independent prognostic factor in acute myeloid leukemia (AML). We improved the identification of this parameter by implementing multiparameter flow cytometry to quantify bone marrow cells carrying leukemia-associated immunophenotypes (LAIP).

Design and Methods. In 106 uniformly treated patients flow cytometric analyses were performed at diagnosis and one week after induction therapy (day 16). The log-difference between LAIP-positive cells on day 1and day 16 (LD16) was determined for each patient.

Results. The LD16 (median, 2.11; range, -0.37 to 4.20) was significantly correlated to CR rate, event-free survival (EFS), overall survival (OS), and relapse-free survival (RFS). Separation of patients by the median LD16 resulted in significant differences in CR rate (81% vs. 51%, p=0.002), EFS (53% at 2 years vs. median 2.8 months, p<0.0001), 2-year OS (58% vs. 43%, p=0.0133), and 2-year RFS (65% vs. 30%, p=0.0037). Multivariate analysis revealed that LD16 was an independent prognostic parameter for CR rate, EFS, and RFS.

Interpretation and Conclusions. Flow cytometric evaluation of early response may serve as a new response parameter in AML. It may be used for development of risk-adapted therapies. High-risk patients can be identified early after the first induction therapy and assigned alternative and salvage treatment strategies.

Key words: acute myeloid leukemia, minimal residual disease, multiparameter flow cytometry, leukemia-associated aberrant immunophenotypes, prognostic factors.

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Acute myeloid leukemia (AML) is a highly aggressive malignant disease resulting from genetic alterations in hematopoietic progenitor cells.[1] The management of patients with AML is based on intensive chemotherapeutic regimens applied for induction and consolidation as well as autologous and allogeneic transplantation procedures. Depending mainly on the karyotype of the disease and the age of the patient 50% to 80% of all individuals achieve a complete remission (CR) and 20% to 50% are cured.[2-4] The main cause of treatment failure is regrowth of minimal residual disease (MRD) which is not detectable by conventional methods such as cytomorphology.

Various prognostic parameters have been defined in patients with AML among which the karyotype of the disease,[5-8] the age of the patient,[9,10] and the secondariness of the disease[11-13] are the most important ones. These parameters can identify patients with a favorable prognosis (e.g. young patients with AML and t(15;17)) as well as of patients with a particularly unfavorable prognosis (e.g. older patients with AML and a complex aberrant karyotype). However, the majority of patients have an intermediate prognosis and no presently available pre-therapeutic parameter predicts the efficacy of standard therapies. Therapy-dependent prognostic parameters may, however, allow the prognosis to be estimated in relation to the response of the disease in the individual patient.

For three decades the cytomorphologic assessment of CR has been the only therapy-dependent parameter in AML which has been used to stratify treatment.[14,15] Thus, patients who achieve a CR receive standard consolidation therapies while patients

without a CR are assigned to salvage regimens. The definition of a CR does, however, have the drawbacks of a low sensitivity (5%), dependence on the individual skills of the morphologist, and the fact that there is a relatively long time between the initiation of therapy and the documentation of non-response. Furthermore, many patients suffer from relapses of the disease despite having achieved a CR, indicating the persistence of treatment-refractory leukemic cells. Several studies have shown that, in subsets of patients with AML in complete remission, genetically or flow cytometrically determined levels of minimal residual disease are related to the patients´ prognosis.[16-23] The present report describes an approach which allows an early and highly sensitive assessment of responsiveness of AML to induction therapy by multiparameter flow cytometry yielding a new, powerful, and independent prognostic parameter.

Design and Methods

AML samples

Fresh bone marrow samples which were sent for reference diagnostics to our laboratory from patients with newly diagnosed and untreated *de novo* or secondary AML following MDS or chemotherapy for other malignancies were immunophenotyped as described below. In all cases cytomorphology, cytochemistry, cytogenetics, and molecular genetics were also applied as detailed below.[24-26] For inclusion in the study a follow-up bone marrow sample had to be sent for early response assessment during aplasia on day 16 after start of TAD-9 induction (day 12 after HAM induction), i.e. one week after completion of the first course of induction therapy. For the sake of simplicity this time point is referred to as day 16 in the following sections of the manuscript.

Antileukemic treatment

All patients were treated within the 1999 trial of the German AML Cooperative Group. Patients older than 16 years of age with newly diagnosed *de novo* or secondary AML were eligible for this trial. Patients with acute promyelocytic leukemia were treated in a separate trial.[27] Patients with severe comorbidity precluding the initiation of intensive induction chemotherapy (i.e., severe uncontrolled infections, coronary heart disease WHO grade III/IV, congestive heart failure WHO grade III/IV, severe hyperbilirubinemia WHO grade III/IV or severe creatinine elevation WHO grade III/IV unless due to leukemia) were excluded.

Patients were treated according to the double induction strategy for remission induction, irrespective of response of the disease to the first course.[28,29] Patients were randomized to receive either the TAD-9 combination[30] or the HAM combination as the first course.[31] The second course of double induction was HAM in all patients. The second course was applied to patients older than 60 years only if they had ≥ 5% residual leukemic blasts in the bone marrow on day 16.

Consolidation therapy consisted of one course of TAD-9. Patients with HLA-identical sibling donors subsequently underwent allogeneic bone marrow or peripheral blood stem cell transplantation. All other patients received further treatment according to the randomization performed at study entry. Patients under the age of 60 were randomized upfront to maintenance therapy or to autologous stem cell transplantation. Patients older than 60 years received maintenance therapy without randomization. Maintenance therapy was applied as described previously.[32,33] Autologous stem cell transplantation was performed after conditioning with busulfan and cyclophosphamide.

Normal bone marrow samples

Normal bone marrow, used as a control, was obtained from healthy volunteers and analyzed by flow cytometry as detailed below.

Flow cytometry

All studies were performed on bone marrow samples. The samples were processed by a Ficoll-Hypaque gradient centrifugation to isolate mononuclear cells both at diagnosis and at day 16.[34,35] Applying triple-staining and isotype controls, monoclonal antibodies against 31 antigens were used in the following combinations designed for the detection of leukemia-associated aberrant immunophenotypes (LAIP) at diagnosis (conjugated with the fluorochromes FITC, PE, and PC-5, respectively): CD11b/CD117/CD34, CD14/CD13/CD4, CD15*/CD34/CD3, CD34/NG2(7.1)/CD33, CD34/CD116/CD33, CD34/CD13/CD19, CD34/CD135/CD117, CD34/CD15*/CD33, CD34/CD19/CD13, CD34/CD2/CD33, CD34/CD56/CD, CD36/CD235a/CD45, CD38/CD133**/CD34, CD38/CD34/CD90, CD4/CD64*/CD45, CD64*/CD4/CD45, CD65/CD87/CD34, CD7/CD33/CD34, CD90/CD117/CD34, HLADR/CD33/CD34,MPO***/LF***/cCD1*,TdT/cCD33/cCD45, TdT/cyCD22/cyCD3, TdT/ cyCD79a/cyCD3.

All antibodies were purchased from Immunotech (Marseilles, France), except for: *Medarex (Annandale, NJ, USA); **Milteny Biotech (Bergisch Gladbach, Germany); ***Caltag (Burlingame, CA, USA). After evaluation of the diagnostic samples, the combinations of antibodies which best covered the LAIP were selected and applied to day 16 samples. The respective combinations of antibodies were added to 10^6 mononuclear cells (volume, 100 μL) and incubated for ten minutes. After addition of 2 mL lysing solution (ammonium chloride-based; prepared at a local pharmaceutical

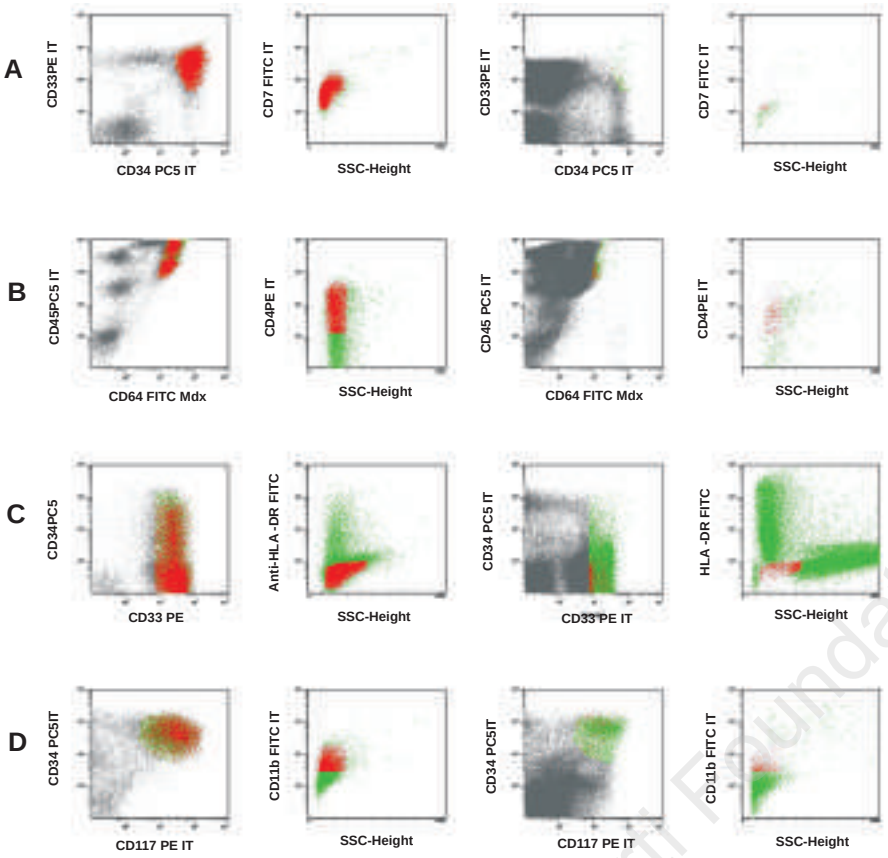

Figure 1. Four different classes of leukemia-associated aberrant immunophenotypes. The 1st and 2nd plots in a row are leukemic bone marrow, the 3rd and 4th plots are normal bone marrow; the 2nd and 4th plots display only cells gated in the 1st and 3rd plots (green cells), respectively; green dots represent cells with immunophenotype as defined in AML by first two antigens, red dots represent cells with immunophenotype as defined in AML by three antigens and OLS-signal; A: cross-lineage antigen expression, $CD33^{+}CD34^{+}$ and $CD7^{+}SSC^{low}$; B: antigen overexpression, $CD45^{++}$ $CD64^{++}$ and $CD4^{+}SSC^{low}$; C: lack of antigen expression, $CD34^{+}$ $CD33^{+}$ and $HLA\text{-}DR\text{-}SSC^{low}$; D: asynchronous antigen expression, $CD34^{+}CD117^{+}$ and $CD11b^{+}SSC^{low}$.

institute) the samples were incubated for an additional 10 minutes and were then washed twice in phosphate-buffered saline (PBS) and resuspended in 0.5 mL PBS. Multiparameter flow cytometry analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Josè, CA, USA). For AML samples at diagnosis 20,000 events were acquired, for day 16 AML samples and for normal bone marrow samples 250,000 events were acquired. Life-gating was not applied. List-mode files were analyzed using the CellQuest Software (Becton Dickinson).

Gating strategy

LAIP were defined individually for each patient by gating on populations displaying an aberrant expression of surface or cytoplasmic antigens and by applying Boolean algebra.[34] LAIP were grouped into 1) asynchronous antigen expression, 2) cross-lineage antigen expression, 3) lack of antigen expression, and 4) antigen overexpression (Figure 1). The combination of gates obtained by this strategy was applied to the list mode files obtained during acquisition of day 16 samples as well as to the list mode files containing the measurements of normal bone marrow samples which had been performed using the same combinations of antibodies. In contrast to other diseases, such as acute lymphoblastic leukemia, which generally display homogeneous populations, many cases of AML present with several leukemic subpopulations within a single sample. Since it is not possible in these cases to include all leukemic cells into one LAIP, the frequencies of LAIP-positive cells are relatively low in these cases (*see Results*). In fact, using this approach in some cases only subpopulations are covered by the respective LAIP. Therefore, the degree of reduction of the leukemic cell mass at day 16 rather than the crude percentage of cells carrying LAIP at day 16 was assessed for prognostic relevance. Thus, for each patient, *a log-difference day 1→day 16* (LD16) was determined, which was defined as the logarithm of the ratio *percentage of LAIP-positive cells at diagnosis/percentage of LAIP-positive cells at day 16*. Accordingly, a reduction of LAIP-positive cells from 30% to 0.3% would result in a LD16 of 2.00 while persistence of 30% LAIP-positive cells would result in a LD16 of 0.00.

In order to estimate the sensitivities of the respective LAIP as well as the ranges in which quantification of the LD16 is feasible, the percentages of LAIP-positive cells within normal bone marrow were determined for each of the applied LAIP. As for the definition of the LD16 for AML blasts, a log-difference to normal bone marrow was calculated which was defined as the logarithm of

the ratio *percentage of LAIP-positive cells in AML sample at diagnosis/median percentage of LAIP-positive cells in normal bone marrow*. Thus, the log-difference to normal bone marrow would equal 3.00 in a case in which LAIP-positive cells formed 50% of the leukemic bone marrow and a median of 0.05% of normal bone marrow. If more than one LAIP was present in a patient, analyses were performed using only the LAIP with the highest log-difference to normal bone marrow in that patient.

In case of a median frequency of 0.00% of LAIP-positive cells in AML samples at day 16 or in normal bone marrow this frequency was set at 0.004% in order to allow the calculation of the respective log-difference (0.004% is the highest frequency displayed as 0.00% by the Cell Quest Pro software and was chosen as the worst case possible). If more than one LAIP was defined in one patient the most sensitive LAIP was selected for the respective evaluations as indicated on the basis of the maximum log difference to normal bone marrow in comparison to other LAIP in the same patient.

Cytomorphology, cytogenetics, molecular genetics

Cytomorphologic assessment was based on May-Grünwald-Giemsa stains, myeloperoxidase reaction, and non-specific esterase using α-naphthyl-acetate as described before.[9,36,37] AML was diagnosed cytomorphologically according to the criteria defined in the FAB classification.[38-40]

Cytogenetic analyses were performed centrally according to standard protocols and the data classified using the ISCN nomenclature.[41-43] Patients were allocated into three subgroups based on cytogenetics: the group considered to have a favorable prognosis included patients with AML with t(8;21), inv(16), or t(16;16); the group with an unfavorable prognosis contained cases of AML with aberrations of chromosomes 5 or 7, aberrations of 11q23 or 17p, inv(3), t(3;3), or with a complex aberrant karyotype (i.e. ≥3 clonal chromosome aberrations); the group associated with an intermediate prognosis included AML patients with other karyotypic aberrations as well as AML patients with a normal karyotype.

Molecular genetic analyses were performed as described previously in detail.[25,44] Samples were analyzed for length mutations of the FLT3 gene (FLT3-LM) as well as mutations around codon D835 of the FLT3 gene.

Study parameters

Bone marrow examinations were carried out on day 16 following TAD-9 induction and on day 12 following HAM induction, i.e. one week after the end of chemotherapy (for determination of LD16), and upon full recovery of peripheral blood counts. Response to therapy was assessed according to CALGB criteria[28,45] as described before.[10] Cases in which the patient died before the LD16 determination were excluded from the present analyses. Relapse, overall survival (OS), event-free survival (EFS), and relapse-free survival (RFS) were defined as described elsewhere.[10]

Statistics

Dichotomous variables were compared between different groups using the χ^2-test and continuous variables by Student´s T-test. The time-dependent variables, OS, EFS, and RFS, were estimated by Kaplan and Meier[46] and differences between the respective groups were calculated using the log rank test. Spearman's rank correlation was used to analyze correlations between continuous parameters. A logistic regression model (dependent variable: CR rate) and Cox models (dependent variables: EFS, OS, and RFS) were used for multivariate analyses. The covariates entered into these models were age, WBC, bone marrow blasts at day 1, bone marrow blasts at day 16, LAIP-positive bone marrow cells at day 16, and LD16 as continuous variables, respectively, as well as favorable cytogenetics, unfavorable cytogenetics, presence of secondary AML, FLT3-LM, and FLT3-D835 mutations as dichotomous variables. All calculations were performed using the SPSS 11.0.1 software (SPSS Inc., Chicago, IL, USA). All *p* values reported are two-sided.

Study conduct

Prior to therapy all patients gave their informed consent to participation in the current evaluation after having been advised about the purpose and investigational nature of the study as well as its potential risks. The study design adhered to the declaration of Helsinki and was approved by the ethics committees of the participating institutions prior to its initiation.

Results

Patients

Between March 2000 and January 2003 bone marrow samples from 106 patients were analyzed both at diagnosis, applying the complete panel of monoclonal antibodies as described above, and at day 16, applying only the selected combination of monoclonal antibodies which allowed the best definition of a LAIP. The patients´ characteristics are given in Table 1. All patients were treated within the 1999 trial of the German AML Cooperative Group as detailed above. The complete remission rate was 63%, the median event-free survival was 7.3 months, the median overall survival was 19.5 months, and the relapse-free survival at 2 years was 53%. Induction therapy was HAM/HAM in

47 patients and TAD/HAM in 59 patients. An allogeneic stem cell transplantation was performed in 24 patients. The outcome of the analyzed patients was not affected by the applied induction and post-remission therapies.

Normal bone marrow

A total of 26 normal bone marrow samples from healthy volunteers were analyzed as controls. The complete panel of monoclonal antibodies was applied.

Leukemia-associated aberrant immunophenotypes (LAIP)

One LAIP was defined for each patient from whom samples at both time points (at diagnosis and at day 16) had been sent to our laboratory (Table 2). The distribution between different classes of LAIP was asynchronous antigen expression (n=23), cross-lineage expression of lymphoid antigens (n=38), lack of antigen expression (n=11), and antigen overexpression (n=34). The median percentage of bone marrow cells which were LAIP-positive in the diagnostic sample was 19.07% (range, 4.20% to 71.45%). The corresponding median percentage of cells which were LAIP-positive in normal bone marrow samples was 0.05% (range: 0.00% to 3.01%). The highest levels were found in cases with antigen overexpression (T-test: p=0.037 for comparison of the antigen overexpression group with all others; Table 2). The resulting median log-difference day 1→normal bone marrow was 2.60 (range, 0.46 to 4.23).

Assessment of early response at day 16

In day 16 follow-up samples from AML patients the median percentage of bone marrow cells which were LAIP-positive was 0.12% (range, 0.00% to 39.52%; Figures 2 and 3). The resulting median LD16 was 2.11 (range, -0.37 to 4.20).

The percentage of bone marrow cells which were LAIP-positive correlated with the cytomorphologically quantified bone marrow blasts in the same respective samples only in cases with bone marrow blast counts higher than 5% (Spearman's rank correlation: r=0.516, p=0.001) but not in cases with lower blast counts (r=0.059, p=0.708).

Figure 3 demonstrates that multiparameter flow cytometric assessment of MRD at day 16 results in a 2 log broader range and a refined quantification as compared to the cytomorphologic assessment of day 16 blasts.

Prognostic impact of log-difference day 1→day 16 (LD16)

As a continuous variable, LD16 was significantly correlated with achievement of complete remission (p=0.001), event-free survival (p<0.0001), overall survival (p=0.003), and relapse-free survival (RFS, p=0.0003, Table 3). Separation of the patients according to the median value of the LD16 (=2.11) resulted in two groups with very different prognoses (CR: 81% vs. 51%, p=0.002; EFS: 53% at 2 years vs. 2.8 months (median), p<0.0001; OS: 58% vs. 43% at 2 years, p=0.0133; RFS: 65% vs. 30% at 2 years, p=0.0037; Figure 4).

Table 1. Patients' characteristics.

Parameter	*n*	*Median*	*Range*
Sex (male/female)	68/38		
Age (years)		54	18 to 79
Cytogenetics			
favorable	18 (17%)		
intermediate	55 (52%)		
unfavorable	33 (31%)		
de novo AML/secondary AML	90/16		
FAB subtype			
M0	5 (5%)		
M1	24 (23%)		
M2	37 (35%)		
M4	13 (12%)		
M4Eo	11 (10%)		
M5a	5 (5%)		
M5b	1 (1%)		
M6	5 (5%)		
n.a.	5 (5%)		
FLT3-LM (yes/no)	15/89		
FLT3-D835 (yes/no)	5/82		
WBC count (×10⁹/L)	15.2	0.6-544.0	
Randomization for induction therapy (TAD+HAM/HAM+HAM)	47/59		
Randomization for post-remission therapy (APBSCT/maint.)*	33/73		
Allogeneic transplantation in CR1	24		
Bone marrow blasts day 1		75%	10-100%
Bone marrow blasts day 16		5%	0-82%
LAIP+ bone marrow cells day	1	19.07%	4.20-71.45%
LAIP+ bone marrow cells day	16	0.12%	0.00-39.52%
LD16		2.11	-0.37-4.20
Median LAIP+ cells in NBM		0.05%	0.00- 3.01%
log-difference day 1→NBM		2.60	0.46-4.23
Maximum LAIP+ cells in NBM		0.34%	0.00- 11.98%

**all patients older than 60 years were scheduled for maintenance therapy without randomization; FLT3-LM: length mutations of the FLT3 gene; FLT3-D835: point mutations of FLT3 at D835; APBSCT=autologous peripheral blood stem cell transplantation; maint.: maintenance therapy; LAIP: leukemia-associated aberrant immunophenotype; LD16: log-difference day 1→day 16; NBM: normal bone marrow.*

Prognostic impact of conventional parameters

The prognostic impact of favorable cytogenetics, unfavorable cytogenetics, AML as secondary disease,

Table 2. Description of leukemia-associated aberrant immunophenotypes.

Class of LAIP	*LAIP*	*% LAIP cells at day 1*	*median % LAIP+ cells in NBM*
Asynchronous expression	CD11b(+)CD117+CD34+	29.09	0.25
	CD11b+CD117+CD34-	6.74	0.03
	CD11b+CD117+CD34-	27.09	0.04
	CD11b+CD117+CD34-	14.41	0.21
	CD11b+CD117+CD34-	4.97	0.08
	CD11b+CD117+CD34-	23.03	0.06
	CD11b+CD117+CD34+	13.47	0.02
	CD11b+CD117+CD34+	9.29	0.07
	CD11b+CD117+CD34+	25.81	0.01
	CD11b+CD117+CD34+	5.56	0.03
	CD11b+CD117-CD34+	13.26	0.83
	CD15+CD34+CD33+	32.63	0.00
	CD15+CD34+CD33+	40.20	0.04
	CD15+CD34+CD33+	56.15	0.07
	CD15+CD34+CD33+	5.28	0.05
	CD34++CD13++CD19-	26.33	0.02
	CD34++CD13++CD19-	18.34	0.00
	CD34+CD116+CD33+	27.71	0.06
	CD34+CD116+CD33+	9.00	0.10
	CD34+CD116+CD33+	11.74	0.17
	CD34+CD116+CD33+	15.43	0.35
	CD34+CD33+CD15+	25.75	0.26
	CD34+CD33+CD15+	10.22	0.06
Cross-lineage expression	CD7+CD33+CD34-	57.03	0.02
	CD10+CD19-CD13+	30.68	0.00
	CD34+CD13+CD19+	44.27	0.02
	CD34+CD13+CD19+	10.58	0.01
	CD34+CD13+CD19+	11.34	0.04
	CD34+CD13+CD19+	10.67	0.00
	CD34+CD2+CD33+	27.43	0.04
	CD34+CD56+CD33+	64.11	0.01
	CD34+CD56+CD33+	12.41	0.07
	CD34+CD56+CD33+	12.49	0.01
	CD34+CD56+CD33+	17.11	0.41
	CD34+CD56+CD33+	4.88	0.34
	CD34+CD56+CD33+	21.96	0.01
	CD34-CD2+CD33+	7.20	0.64
	CD34-CD56+CD33+	15.90	0.04
	CD34-CD56+CD33+	9.10	0.02
	CD34-CD56+CD33+	25.26	0.01
	CD34-CD56+CD33+	13.77	0.01
	CD4+CD13+CD14-	7.17	0.06
	CD4+CD13+CD14-	20.37	0.13
	CD34(+)CD56+CD33(+)	63.66	0.02
	CD7+CD33+CD34-	16.04	0.04
	CD7+CD33+CD34-	22.57	0.25
	CD7+CD33+CD34-	27.91	0.02
	CD7+CD33+CD34+	27.82	0.13
	CD7+CD33+CD34+	63.44	0.05
	CD7+CD33+CD34+	9.93	0.02
	CD7+CD33+CD34+	32.70	0.00
	CD7+CD33+CD34+	35.12	0.04
	CD7+CD33+CD34+	21.70	0.02
	CD7+CD33+CD34+	56.11	0.03
	CD7+CD33+CD34+	15.60	0.09
	CD7+CD33+CD34+	19.16	0.01
	CD7+CD33+CD34+	12.58	0.05
	CD7+CD33+CD34+	32.48	0.02
	CD7+CD33+CD34+	15.21	0.01
	CD7+CD33+CD34+	18.98	0.08
	CD7+CD33+CD34+	6.16	0.28
Lack of expression	HLA-DR+CD33-CD34+	52.55	0.28
	CD38+CD133-CD34+	5.86	0.01
	HLA-DR-CD33+CD34-	26.96	0.17
	HLA-DR-CD33+CD34-	19.25	0.01
	HLA-DR-CD33+CD34-	21.99	0.19
	HLA-DR-CD33+CD34+	16.76	0.05
	HLA-DR-CD33+CD34+	32.98	0.18
	HLA-DR-CD33+CD34+	51.18	0.10
	HLA-DR-CD33+CD34+	68.42	0.00
	MPO+LF-cCD15-	14.78	0.00
	MPO+LF-cCD15-	30.58	0.01

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Overexpression	CD15+CD34+CD33+	4.75	0.06
	HLA-DR+CD33++CD34-	11.09	1.14
	CD34++CD13++CD19-	12.41	0.03
	CD34+7.1++CD33+	7.31	0.03
	CD34+7.1++CD33+	27.34	0.14
	CD34+7.1++CD33+	43.77	0.03
	CD34+CD56-CD33++	11.21	0.19
	CD34-CD117+CD135(+)	49.84	0.04
	CD36(+)CD235a++CD45(+)	47.77	0.12
	CD36++CD235a++CD45+	9.76	0.08
	CD36++CD235a+CD45+	64.03	0.01
	CD36++CD235a-CD45+	9.97	0.14
	CD38++CD133++CD34++	71.45	0.08
	CD38+CD34++CD90(+)	62.95	0.01
	CD38+CD34++CD90+	12.18	0.32
	CD64++CD4++CD45++	7.43	0.45
	CD64++CD4++CD45++	4.20	0.00
	CD64++CD4++CD45++	32.55	0.01
	CD64++CD4++CD45++	15.38	0.04
	CD64++CD4++CD45++	30.84	0.29
	CD64++CD4++CD45++	25.33	0.20
	CD64++CD4-CD45++	24.92	0.05
	CD65++CD87++CD34-	6.32	0.02
	CD65++CD87++CD34-	40.54	0.17
	CD65++CD87++CD34-	9.44	1.05
	HLA-DR+CD33++CD34++	5.25	0.12
	TdT(+)cCD33++cCD45++	10.18	0.01
	TdT(+)cCD33++cCD45++	10.76	0.12
	TdT(+)cCD33++cCD45++	9.70	0.04
	TdT(+)cCD33++cCD45++	21.34	0.01
	TdT-cCD33++cCD45++	32.87	0.00
	TdT-cCD33++cCD45++	48.30	0.11
	TdT-cCD33++cCD45++	8.71	3.01
	TdT-cCD33++cCD45++	35.28	0.10
Asynchronous expression (n=23)	(median, range)	15.43 (4.97-56.15)	0.06 (0.00-0.83)
Cross-lineage expression (n=38)	(median, range)	19.07 (4.88-64.11)	0.03 (0.00-0.64)
Lack of expression (n=11)	(median, range)	26.96 (5.86-68.42)	0.05 (0.00-0.28)
Overexpression (n=34)	(median, range)	13.90 (4.20-71.45)	0.08 (0.00-3.01)

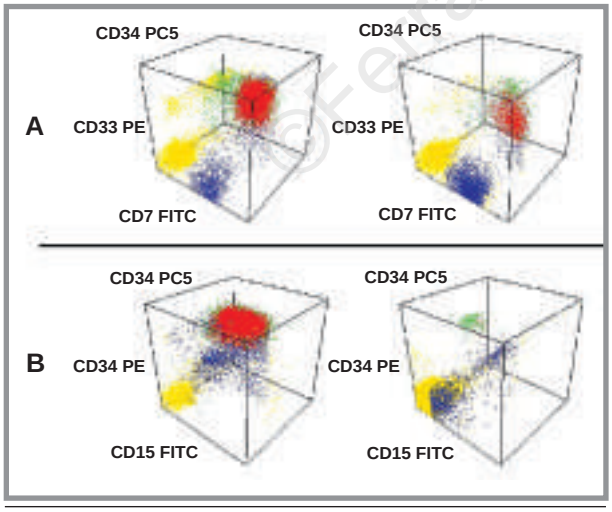

Figure 2. Identification of MRD on day 16. The 1st and 2nd plots in a row are analyses at diagnosis and at day 16, respectively. A: Patient with aberrant cross-lineage expression of CD7 on cells positive for CD33 and CD34; residual leukemia is detected at day 16. B: Patient with aberrant asynchronous expression of CD15 on cells positive for CD33 and CD34; no residual leukemia is detected at day 16.

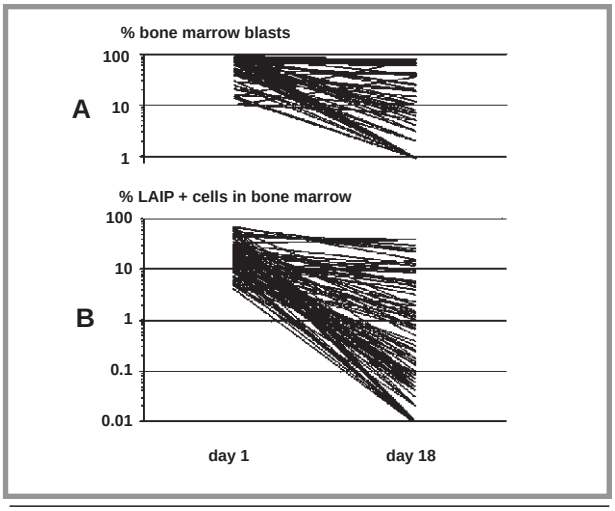

Figure 3. Decrease in leukemic cell mass as assessed by cytomorphology (A) and by multiparameter flow cytometry (B). The cytomorphologic evaluation results in a 2-log distribution at the response checkpoint on day 16 and tends to result in categorized data such as 0%, 5%, and 10%. The flow cytometric evaluation results in a nearly 4-log distribution at day 16 and shows, due to the automation, no tendency towards a categorization of results.

Table 3. Univariate analyses.

	CR		EFS		OS		RFS	
	p	*RR (95%-CI)*	*P*	*RR (95%-CI)*	*p*	*RR (95%-CI)*	*p*	*RR (95%-CI)*
Favorable karyotype	0.039	5.037 (1.089-23.293)	0.003	0.167 (0.052-0.538)	0.030	0.204 (0.048-0.855)	0.021	0.094 (0.013-0.704)
Unfavorable karyotype	0.0002	0.182 (0.075-0.445)	<0.0001	4.200 (2.412-7.312)	<0.0001	4.247 (2.121-8.501)	0.001	4.837 (1.929-12.127)
Age	0.135	0.979 (0.953-1.007)	0.086	1.016 (0.998-1.034)	0.218	1.014 (0.992-1.037)	0.385	1.012 (0.986-1.038)
WBC	0.342	1.554 (0.412- 11.697)*	0.865	1.035 (0.630-1.44)*	0.085	1.313 (0.957-1.668)*	0.061	1.409 (1.000- 1.836)*
sAML	0.013	0.244 (0.080-0.740)	0.0003	3.360 (1.735-6.508)	0.003	3.271 (1.507-7.099)	0.001	11.281 (2.775-45.867)
FLT3-LM	0.236	2.246 (0.590-8.552)	0.587	1.220 (0.596-2.497)	0.427	1.433 (0.590-3.481)	0.023	3.043 (1.168-7.927)
FLT3-D835	0.523	2.074 (0.221-19.449)	0.214	0.284 (0.039-2.065)	0.863	0.880 (0.207-3.747)	0.430	0.044 (0.000-103.305)
BM blasts day 1	0.837	1.002 (0.986-1.018)	0.634	1.003 (0.992-1.014)	0.922	0.999 (0.985-1.013)	0.861	1.002 (0.984-1.019)
BM blasts day 16	0.050	0.982 (0.964-1.000)	0.002	1.016 (1.006-1.026)	0.056	1.012 (1.000-1.025)	0.064	1.015 (0.999-1.031)
LAIP+ BM cells day 16	0.027	0.933 (0.878-0.992)	0.0002	1.053 (1.025-1.082)	0.033	1.036 (1.003-1.069)	0.007	1.076 (1.020-1.135)
LD16	0.001	1.888 (1.287-2.770)	<0.0001	0.535 (0.415-0.690)	0.003	0.635 (0.469-0.859)	0.0003	0.419 (0.263-0.668)

*CR: complete remission; EFS: event-free survival; OS: overall survival; RFS: relapse-free survival; RR: risk ratio; FLT3-LM: length mutations of the FLT3 gene; FLT3-D835: point mutations of FLT3 at D835; LAIP: leukemia-associated aberrant immunophenotype; BM: bone marrow; *per 100×10⁹/L; LD16: log-difference day 1→day 16.*

FLT3-LM, and D835 mutations of the FLT3 gene as dichotomous variables as well as of age, WBC count at diagnosis, percentage of bone marrow blasts at diagnosis, and percentage of bone marrow blasts at day 16 as continuous variables was analyzed using achievement of CR, EFS, OS, and RFS as dependent variables. Favorable and unfavorable cytogenetics as well as AML as secondary disease were significantly related to all tested dependent variables. Table 3 details the results of these *univariate* analyses.

Multivariate analysis of prognostic parameters

All prognostic parameters which were identified in univariate analyses to carry significant prognostic impact were further evaluated in multivariate analyses. The three therapy-dependent parameters, day 16 bone marrow blasts, LAIP-positive bone marrow cells at day 16, and LD16, were included into a logistic regression model and into a Cox model, respectively, to analyze for the anticipated dependences of these variables. LD16 proved to be the only independent prognostic parameter (CR: p=0.145; EFS: p=0.007; OS: p=0.127; RFS: p=0.009) and was subsequently included into multivariate analyses while day 16 bone marrow blasts and LAIP-positive bone marrow cells at day 16 were not.

A logistic regression model was used to evaluate the achievement of CR and Cox models were used for the evaluation of EFS, OS, and RFS (Table 4).

Supporting the concept that LD16 is an early *in vivo* indicator of chemosensitivity, the results of these analyses show that the LD16 is the most important prognostic parameter being for EFS (the only other significant parameter, unfavorable cytogenetics) and the only independent parameter for RFS. Unfavorable cytogenetics were the only independent parameter with impact on OS. Both unfavorable cytogenetics and LD16 were parameters independently influencing the CR rate.

Prognostic impact of LD16 in subgroups defined by a cut-off of 5% day 16 bone marrow blasts

To determine whether the prognostic impact of the LD16 was limited to cases with ≤5% day 16 bone marrow blasts and whether it could add to the prognostic power of the day 16 bone marrow blasts if these accounted for more than 5% of the cellularity, two

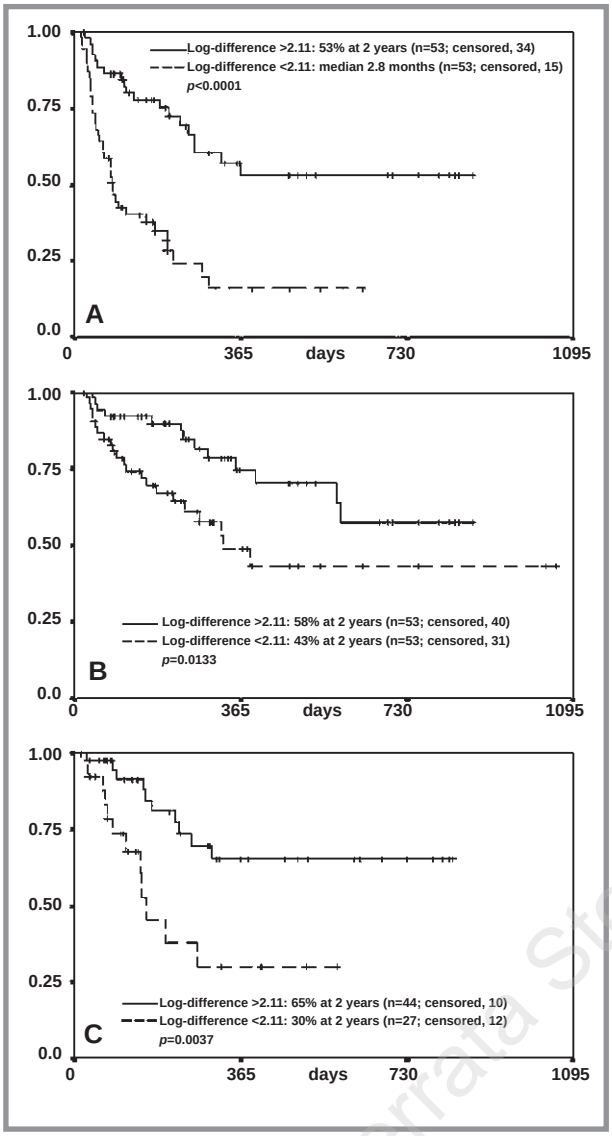

Figure 4. Separation of patients into two groups according to the median log-difference day 1→day 16 (median=2.11). The separation results in significant differences for EFS (A), OS (B), and RFS (C).

analyses were performed with patients separated into two groups, one with ≤5% and the other with >5% day 16 bone marrow blasts (Table 5). It is evident that, as a continuous variable, the LD16 had a significant impact on EFS and RFS in cases with ≤5% day 16 bone marrow blasts and also tended to influence the achievement of CR and the OS. In cases with >5% day 16 bone marrow blasts the LD16 was significantly correlated to all end-points. To define the respective roles of the LD16 and the day 16 bone marrow blasts further, multivariate analyses of EFS were performed within the identical two groups taking into consideration both parameters. These analyses confirmed that the LD16 had independent prognostic power in both groups of patients with either ≤5% or more than 5% day 16 blasts (p=0.020 and p=0.014), while the day 16 blasts did not (p=0.293 and p=0.401).

Prognostic impact of LD16 in cytogenetically defined subgroups

In order to further strengthen the independent prognostic power of the LD16, analyses within cytogenetically defined subgroups were performed (favorable, intermediate, and unfavorable cytogenetics) (Table 6). The median LD16 values and their ranges within these three groups were 2.73 (0.45 to 3.91), 2.34 (0.12 to 4.20), and 1.20 (-0.37 to 4.15). There were strong trends for relations between the LD16 and CR rate, EFS, and RFS within all cytogenetically defined subgroups although a signficant relation was found only for EFS and RFS in the group with intermediate cytogenetics.

Prognostic impact of LD16 in subgroups defined by class of LAIP

To analyze whether the prognostic impact of LD16 was influenced by the LAIP class, all dependent variables were assessed within the four classes of LAIP (Table 7). There were significant relations to EFS in all LAIP classes and strong trends for relations to the oth-

Table 4. Multivariate analyses.

	CR		*EFS*		*OS*		*RFS*	
	p	*RR (95%-CI)*	*P*	*RR (95%-CI)*	*p*	*RR (95%-CI)*	*p*	*RR (95%-CI)*
Favorable karyotype	0.367	2.111 (0.416-10.715)	0.044	0.289 (0.086-0.969)	0.170	0.351 (0.079-1.567)	0.101	0.175 (0.022-1.401)
Unfavorable karyotype	0.032	0.330 (0.120-0.909)	0.007	2.293 (1.251-4.205)	0.021	2.604 (1.157-5.863)	0.115	2.421 (0.806-7.271)
sAML	0.435	0.612 (0.179-2.098)	0.280	1.466 (0.732-2.936)	0.406	1.442 (0.608-3.417)	0.198	2.824 (0.582-13.707)
FLT3-LM	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.215	1.996 (0.670-5.945)
LD16	0.062	1.490 (0.980-2.265)	0.004	0.678 (0.519-0.885)	0.355	0.852 (0.608-1.196)	0.031	0.555 (0.325-0.948)

CR: complete remission; EFS: event-free survival; OS: overall survival; RFS: relapse-free survival; RR: risk ratio; FLT3-LM: length mutations of the FLT3 gene; n.a.: not applicable; LD16: log-difference day 1→day 16.

Table 5. Impact of log-difference day 1→day 16 (LD16) on outcome in subgroups defined by a cut-off of 5% day 16 bone marrow blasts.

	CR		EFS		OS		RFS	
	p	*RR (95%-CI)*	*P*	*RR (95%-CI)*	*p*	*RR (95%-CI)*	*p*	*RR (95%-CI)*
day 16 blasts ≤5%	0.169	1.642 (0.810-3.331)	0.025	0.601 (0.385-0.940)	0.186	0.686 (0.392-1.200)	0.023	0.382 (0.166-0.877)
day 16 blasts >5%	0.014	1.800 (1.126-2.875)	0.0001	0.531 (0.385-0.733)	0.023	0.642 (0.439-0.940)	0.005	0.428 (0.235-0.779)

CR: complete remission; EFS: event-free survival; OS: overall survival; RFS: relapse-free survival; RR: risk ratio.

Table 6. Impact of log-difference day 1→day 16 (LD16) on outcome in cytogenetically defined subgroups.

Subgroup	CR		EFS		RFS	
	p	*RR (95%-CI)*	*P*	*RR (95%-CI)*	*p*	*RR (95%-CI)*
Favorable karyotype (n=18)	0.099	6.530 (0.702-60.767)	0.162	0.367 (0.090-1.498)	0.368	6.024 (0.121-299.493)
Intermediate karyotype (n=55)	0.121	1.567 (0.888-2.766)	0.010	0.603 (0.410-0.888)	0.036	0.528 (0.290-0.961)
Unfavorable karyotype (n=33)	0.584	1.195 (0.632-2.261)	0.187	0.780 (0.540-1.128)	0.152	0.553 (0.246-1.244)

CR: complete remission; EFS: event-free survival; RFS: relapse-free survival; RR: risk ratio.

Table 7. Impact of log-difference day 1→day 16 (LD16) on prognosis in subgroups defined by class of LAIP.

Class of LAIP	CR		EFS		OS		RFS	
	p	*RR (95%-CI)*	*P*	*RR (95%-CI)*	*p*	*RR (95%-CI)*	*p*	*RR (95%-CI)*
asynchronous expression (n=23)	0.025	2.582 (1.125-5.923)	0.010	0.425 (0.222-0.817)	0.264	0.596 (0.240-1.478)	0.379	0.007 (0.000-416.665)
cross-lineage expression (n=38)	0.153	1.568 (0.847-2.904)	0.021	0.604 (0.394-0.925)	0.064	0.653 (0.416-1.025)	0.034	0.390 (0.163-0.930)
lack of expression (n=11)	0.083	7.567 (0.771-74.316)	0.039	0.159 (0.028-0.907)	0.219	0.397 (0.091-1.732)	0.253	0.100 (0.002-5.180)
overexpression (n=34)	0.340	1.380 (0.712-2.676)	0.048	0.656 (0.432-0.997)	0.053	0.535 (0.284-1.007)	0.083	0.588 (0.323-1.071)

CR: complete remission; EFS: event-free survival; OS: overall survival; RFS: relapse-free survival; RR: risk ratio.

er dependent variables. Strengthening the concept that MRD assessment by multiparameter flow cytometry could be usefully applied to all cases with AML, there was absolutely no indication that relations of the LD16 to prognosis would be inferior for the LAIP class with the lowest level of LAIP-positive cells in the leukemic bone marrow and the highest level of LAIP-positive cells in the normal bone marrow (i.e. the class with overexpression of antigens) than they would be for the other classes of LAIP.

Discussion

The present data demonstrate that the multiparameter flow cytometric assessment of MRD is feasible and clinically useful in patients with AML. This assessment results in a powerful and independent prognostic parameter which can be used as a basis for early treatment stratification. The present data are particularly important because of a) the early checkpoint during aplasia following induction therapy, b) the methodological applicability of the approach to all unselected patients with AML, and c) the comprehensive multivariate analysis with inclusion of complete data sets on cytogenetics, secondary AML, and other parameters in a population with no upper age limit.

The only established and clinically accepted therapy-dependent prognostic parameter which is broadly used for treatment stratification has been the achievement of CR.[47] Patients not achieving a CR have a poorer prognosis and are assigned to salvage regimens. Although

this parameter is very powerful it has the drawback of being evaluated relatively late, several weeks after the start of therapy. In contrast, recent data indicate that the identification of patients with refractory disease is possible as early as one week after the end of the first course of induction therapy.[10] Following pivotal studies which provided the basis for the concept of assessing residual leukemic bone marrow blasts on day 16 as a surrogate *in vivo* marker for chemosensitivity,[48-50] day 16 blasts were proven to be an independent prognostic parameter in a large prospective study.[10] This parameter has been assessed even two weeks earlier than the bone marrow CR as defined by the British MRC which was also shown to have a significant impact on prognosis.[51] Interestingly, the parameters in both studies were most powerful in patients with an otherwise intermediate prognosis but had no prognostic impact in patients with CBF leukemias.[10,51] The MRC definition of CR, which differs from the CALGB and NCI criteria by the lack of a full recovery of peripheral blood counts, already aimed at defining the therapy-dependent prognosis very early in order to allow a timely adaptation of therapy. This is in line with data on the lack of prognostic impact of achievement of 1,500/μL neutrophils as well as of the presence of peripheral blasts during the remission evaluation of bone marrow.[52] Overall, these data strongly argue in favor of an early bone marrow evaluation for response to therapy in AML to yield a powerful and clinically applicable prognostic parameter. The present study provides a substantial improvement of these studies and of the parameter, day 16 blasts, in particular. Thus, we show that the LD16 is prognostically more relevant than the morphologically evaluated day 16 blasts. Accordingly, a prognostic impact was demonstrated for both cases with ≤5% and >5% day 16 blasts. This result was achieved using multiparameter flow cytometry, which has both higher sensitivity and greater reproducibility than cytomorphology. These data suggest that assessment of day 16 blasts, despite its prognostic usefulness, should be replaced by the multiparameter flow cytometric quantification of disease response.

Previous studies dealing with the flow cytometric detection and quantification of MRD in AML demonstrated that MRD levels after achievement of CR and following consolidation therapy are highly predictive of the patients´ outcome.[21-23] However, these studies only included patients in whom a highly aberrant LAIP had been identified. Thus, 25% to 40% of the cases had to be excluded from the analyses due to leukemic cells not displaying a LAIP as aberrant as required in the respective studies. These excluded cases amounted to only 20% in further reports, however, these reports did not put their data into the context of clinical follow-up assessment and the analysis of the prognostic impact of flow cytometrically detected MRD levels.[20,53-56] The present report describes a different approach to this issue by anticipating that it is possible to define a LAIP in all AML patients by using a large panel of triple combinations of antibodies and by analyzing the prognostic significance of MRD levels obtained in this way. In fact, a LAIP was identified in all of the unselected samples sent to the laboratory at diagnosis and on day 16 and the LD16 independently predicted the probability of achieving a CR as well as EFS and RFS. Moreover, analyses in subgroups indicated that the prognostic value was not limited to highly aberrant LAIP but was also present for less aberrant LAIP which were excluded from previous studies.[21-23] Thus, the group of AML displaying an overexpression of an antigen has been anticipated to reveal the least sensitive LAIP and, in fact, cases within this group displayed LAIP which were present in significantly more cells within normal bone marrow than all other cases. Nonetheless, even in this group there were significant relations between the LD16 and EFS, OS, and RFS. This is further evidence that flow cytometrically based MRD detection and quantification could be useful in the vast majority of patients with AML.

In contrast to previous reports on immunologic monitoring of AML,[21-23] no distinct level of MRD was identified to carry prognostic importance. While in these other reports, most of the analyzed cases had more than 50% LAIP-positive cells in the bone marrow at diagnosis, in our study this percentage ranged from 4.20% to 71.45% due to the inclusion of unselected patients with AML. The percentage of LAIP-positive cells at day 16 is influenced not only be the degree of leukemic cell mass reduction but also by the percentage of LAIP-positive cells at diagnosis. However, it has been shown that the LD16 is prognostically more important than the absolute percentage of LAIP-positive cells at day 16. Thus, when applying immunologic monitoring to unselected cases of AML the degree of leukemic cell mass reduction should be used for prognostication rather than the percentage of LAIP-positive cells.

The present data are of particular value with regard to the analyses of the prognostic impact of the LD16 together with a complete data set on the karyotype aberrations of the leukemic cells in each patient. In previous analyses, data on cytogenetics were available for only 60% to 88% of the cases thus leaving open the question of whether MRD levels would have a prognostic impact in addition to that of the karyotype aberrations or whether they would just reflect these aberrations.[21-23] The present report demonstrates that the LD16 independently influences the probability of achieving a CR and the EFS and the RFS rates. Accordingly, there are strong relations between these parameters within cytogenetically defined risk-groups although they were significant only in the group with

prognostically intermediate karyotypes. It was expected that the influence of the LD16 on OS would be less strong since the LD16 is an *in vivo* surrogate marker for chemosensitivity and thus not suited to reflecting OS achieved by the 24 patients who received an allogeneic transplantation. As a consequence, it was possible to separate all patients according to the median LD16, which resulted in two groups with significantly differing OS, however, the LD16 did not add prognostic information independent from that offered by the presence of an unfavorable karyotype.

Although the LD16 already is a very powerful prognostic parameter, its strength may be further enhanced by technical improvements, such as the use of four flourescence dyes and the introduction of CD45 gating,[57] as well as by the detection of new proteins which are aberrantly expressed in leukemic cells.[35,58] Overall, the concept of the LD16 as presented in this report provides the impetus and basis for starting a revision of response assessment in patients with AML, with a view to improving the individual and risk-adapted management of this disease.

WK: principal investigator, DV: contribution to conducting the work and interpreting results, CS: contribution to conducting the work and interpreting results, SS: contribution to conducting the work and interpreting results, WH: contribution to interpreting results, TH: contribution to conducting the work and interpreting results. All authors contributed to the design of the study and the revision of the manuscript. Primary responsibility for the publication and for each Table and Figure: WK.

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